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PREFACE

Once again it is our pleasurable opportunity to thank the many who have collaborated in preparing this present volume of the *Annual Review of Biochemistry.* All of the manuscripts were received; there were no last-minute withdrawals. For this unanimity in response we are especially grateful.

Some manuscripts were unusually late. Unavoidable difficulties were encountered at the Press. This combination of events led to an unfortunate delay in publication. We are increasingly hopeful that within another year or two prompt publication of the *Review* may again be possible.

The usual difficulties attendant upon the war have again been experienced in preparing this present volume. They need not be recited, for to all of us, authors and readers alike, they are quite familiar. In view of the continuing inaccessibility of some foreign journals we would venture to renew our appeal for reprints, especially from our colleagues abroad; these will be distributed to the authors of forthcoming reviews.

Again we would express our gratitude to Professor H. S. Loring, to our editorial assistants, to our office staff generally, and to the Stanford University Press for their fine co-operation throughout.

H.J.A. C.L.A.S.
D.R.H. J.H.C.S.
J.M.L. H.A.S.

ERRATA

Volume XII, page 34, line 10 from bottom: *for* carbon suboxide (C_2O_3), *read* carbon suboxide (C_3O_2).

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BIOLOGICAL OXIDATIONS AND REDUCTIONS

By D. E. Green and P. K. Stumpf

Departments of Medicine and Biochemistry, College of Physicians and Surgeons, Columbia University, New York

Iron Porphyrin Protein Enzymes

There has been a revival of interest in the study of peroxidase after a lull of some years. Agner (1) working with empyema fluid of tuberculous patients succeeded in isolating the so-called verdoperoxidase of leucocytes in highly purified form. The final product with a pronounced green color contained 0.1 per cent iron and about 1 per cent hemin. It was homogeneous as determined both in the ultracentrifuge and in the Tiselius apparatus.

The catalytic activity of verdoperoxidase per mole of hemin is less than 1/10 of that of peroxidase prepared from horse radish. In addition the two enzymes have different absorption spectra. However, both form compounds with hydrogen peroxide, hydrogen cyanide, sodium azide, and hydroxylamine, and catalyze the oxidation by hydrogen peroxide of much the same types of organic compounds.

Theorell & Åkeson (2) have purified milk peroxidase to the point where it is probably homogeneous. The best preparations contained about 0.076 per cent iron. The absorption spectrum was not identical either with that of verdoperoxidase or peroxidase prepared from horse radish. The catalytic activity, however, was fairly close to that of verdoperoxidase. On reduction with sodium hydrosulfite the brownish green solution turned a pure green color. The α and β absorption bands of the pyridine hemochromogen formed from milk peroxidase did not correspond with those of protohemochromogen, being shifted 5 and 10 Å respectively toward the red end of the spectrum. Compounds with hydrogen cyanide, hydrogen peroxide, and fluoride were also formed by the milk enzyme as shown by changes in absorption spectrum.

Theorell (3) carrying the method of Keilin & Mann for the purification (4) of horse radish peroxidase some steps further has succeeded in isolating the enzyme in homogeneous and crystalline state. The homogeneity was demonstrated in the cataphoresis apparatus and by test of solubility in ammonium sulfate solutions. The hemin content of the final preparation was 1.48 per cent. The molar paramagnetic susceptibility of peroxidase and some of its derivatives were

measured and it was concluded that despite the spectral similarity of peroxidase and ferrihemoglobin noteworthy differences exist in regard to their magnetic properties.

To date four distinct peroxidases have been isolated, viz. (*a*) horse radish peroxidase, (*b*) verdoperoxidase, (*c*) milk peroxidase, and (*d*) cytochrome-*c* peroxidase. The last mentioned isolated from yeast (5) is unique in that it catalyzes the oxidation by hydrogen peroxide of ferro cytochrome-*c* only. The other three peroxidases attack a great variety of substrates including aromatic amines, phenols, diamines, ascorbic acid, and ferrocytochrome-*c*. Sumner & Gjessing (6) have reported variations in the properties of plant peroxidases. Apparently the peroxidases from horse radish and milk weed have different affinities for hydrogen peroxide. These are probably minor differences like those which exist between the hemoglobins of different animal tissues.

Theorell *et al.* (7) have extended their experiments on the reversible resolution of horse radish peroxidase. The protein moiety was separated from the prosthetic group by precipitation with acetone in dilute solutions of hydrochloric acid. The prosthetic group remained in solution. The split protein combined with protohemin, mesohemin, and deuterohemin, respectively, to form compounds with 100, 53, and 62 per cent of the original activity. Other hemins formed compounds with the specific protein but without catalytic activity. The time for complete combination varied from a few minutes to twenty-four hours. The rate of combination could be followed by the change in spectrum which accompanies the formation of the hemin protein compound. The new peroxidases formed respectively by meso- and deuterohemin had different absorption bands from the natural peroxidase which contains protohemin. The previous review has already dealt with the synthesis of active peroxidases in which metals other than iron are present in combination with protoporphyrin (8). It appears therefore that peroxidase from horse radish is less specific as far as the components of the prosthetic group are concerned than hemoglobin, although both contain the same prosthetic group.

Chance (9, 10) has made a very notable contribution to the kinetics of peroxidase and indeed to the fundamental theory of enzyme kinetics. He has perfected a micro modification of the Hartridge-Roughton flow apparatus for studying the kinetics of rapid enzyme reactions. Enzyme and substrate were mixed rapidly in a suitable flow chamber; the changes in light absorption which accom-

pany chemical reaction were picked up by photocells and the progress of the reaction was measured directly by the photoelectric amplifiers. Mirror oscillograph recordings were made of each kinetic run. He found that horse radish peroxidase forms a compound with hydrogen peroxide with extreme rapidity ($k_1 = 1.2 \times 10^7$ liter mole^{-1} sec.$^{-1}$). This rate is similar to the measured value for the reaction of oxygen with muscle hemoglobin (1.9×10^7 liter mole^{-1} sec.$^{-1}$). The equilibrium for the enzyme-substrate combination favors almost complete association ($K = 2 \times 10^{-8} M$). Chance's data substantiate the conclusion that a second order combination of enzyme and substrate is followed by a first order decomposition reaction. The rate of breakdown of the intermediate compound of peroxidase and hydrogen peroxide in presence of an acceptor like leuco malachite green is small compared to the rate of synthesis. For a particular concentration of malachite green k_3 was found to be 4.2 sec.$^{-1}$. The kinetics of peroxidase indicate that a chain mechanism plays no significant role, if any, in the mode of action of peroxidase. There was some indirect evidence that the acceptor forms a compound with the enzyme-substrate compound. These experiments of Chance provide the first direct experimental proof of the classical Michaelis theory of enzyme action.

The structure and mode of action of catalase are still being actively investigated. Agner (11) has prepared crystalline catalase from horse liver by a new method. His best preparations contained 0.093 per cent iron, 0.8 to 0.9 per cent hemin, and ca. 0.02 per cent copper. Only 75 to 85 per cent of the total iron was bound to hematin. Agner confirmed the presence of bile pigment in the catalase molecule in the ratio of 1 bile pigment to 3 hemins. He is of the opinion that the bile pigment is a natural constituent of the enzyme and that it does not arise by degradation or oxidation of one of the hemins in the catalase molecule during the isolation procedure. Lemberg (12) has championed the theory that the bile pigment of catalase is a degradation product of hemin. His main evidence is that bilirubin is formed by oxidation of hemoglobin in presence of ascorbic acid as catalyst. Perhaps a more cogent line of evidence is the observation of Agner (13) that catalase isolated from erythrocytes does not contain bile pigment and that the catalytic activity of erythrocyte catalase per mole of enzyme is some 50 per cent higher than that of liver catalase.

Theorell & Agner (14) have made paramagnetic susceptibility measurements of horse liver catalase and its derivatives, the results

of which they believe render unlikely the theory of Keilin & Hartree (15) that hydrogen peroxide reduces the hemin iron of catalase from the ferric to the ferrous condition. The present usefulness of paramagnetic measurements can now be put to a decisive test on the issue whether hydrogen peroxide-azide catalase is in the ferrous state as Keilin & Hartree believe or in the ferric state according to the magnetic measurements. In that connection Keilin & Hartree (16) have withdrawn their claim that catalase action does not proceed under anaerobic conditions. One of the supports for the ferrous hypothesis is thereby removed.

Haas (17) has made an interesting attempt to render soluble the cytochrome oxidase. He has found that the oxidase occurs in two forms as prepared by the usual method of grinding washed heart tissue in a mechanical mortar. One form is completely sedimented by a force of 10,000 x g while the other form is not appreciably sedimented. Exposure of such a mixed preparation to ultrasonic radiation increases the proportion of oxidase which is not sedimented by 10,000 x g. It appears that such treatment, like prolonged grinding with sand, leads to a progressively smaller particle size. However, the pronounced Tyndall effect reported by Haas for solutions of the oxidase after centrifugation at 10,000 x g militates against the assumption that the enzyme has been separated from its association with particles.

While ferritin, the ferric hydroxide protein of liver, spleen, and other organs, is not strictly germane to a review of biological oxidations, it has enough interesting possibilities to justify some reference to it here (18 to 21). Under appropriate conditions, ferritin can be split into an iron-free protein which has the same crystalline structure as the parent compound. This so called "apoferritin" has now been obtained in a homogeneous state in contrast to the inhomogeneity of the best ferritin preparations. From magnetic susceptibility measurements Michaelis & Granick concluded that ferric hydroxide is present in "micelles which fill the interstices of the structure of ferritin." Experiments with radioactive iron suggest that ferritin is a storage form of iron in the body.

Flavoprotein Enzymes

With the discovery of glycine oxidase by Ratner *et al.* (22) the number of flavoproteins described up to now has swelled to ten. This enzyme which is widely distributed in liver and kidney of many species catalyzes the oxidation of glycine to glyoxylic acid (CHOCOOH) and ammonia, and of sarcosine to glyoxylic acid and methylamine.

Glycine oxidase as found under normal conditions in liver and kidney is a conjugated flavoprotein containing flavin adenine dinucleotide (FAD). When isolated from lamb, cat, and human kidneys by precipitation with salt within the range of pH 4 to 9 the enzyme does not dissociate appreciably. It is only when the enzyme is exposed to 0.1 *N* hydrochloric acid that dissociation ensues. However, when the enzyme is prepared from pig kidney by the same procedure as used in the preparation of the enzyme from the other kidneys, it no longer behaves as a conjugated flavoprotein but appears to be largely dissociated. Thus after two precipitations with ammonium sulfate no activity was shown except in the presence of added FAD. Analyses showed that this was not a genuine dissociation but that a factor in pig kidney presumably enzymic in nature was responsible for the cleavage of the flavoprotein into its constituent parts. Whether this cleavage was the result of destruction of FAD has yet to be clarified. When adequate precautions were taken to prevent this prosthetic group splitting factor from acting, preparations of the glycine oxidase could be made from pig kidney which failed to show any appreciable dissociation during salt precipitation over the pH range 4 to 9.

Precisely the same considerations were found to apply to the *d*-amino acid oxidase (22). When prepared from pig kidney the enzyme appeared to be a dissociating flavoprotein, whereas the enzyme from lamb kidney was split only under strongly acid conditions. Again when steps were taken to minimize the action of the prosthetic group splitting factor, the preparation from pig kidney showed no tendency to dissociate whatsoever over the pH range 4 to 9.

Unfortunately, practically all the early work on *d*-amino acid oxidase was carried out on preparations from pig kidney. The conclusion that the *d*-amino acid oxidase is almost completely dissociated at neutral pH was based on the analysis of so-called dissociation curves which relate rate of oxidation as a function of added FAD in presence of a fixed amount of the split protein (23, 24). It now appears that these curves were determined largely by rates of combination and therefore could not be used to calculate thermodynamic dissociation constants.

Fischer *et al.* (25) have made more observations on fumaric hydrogenase, the flavoprotein which catalyzes the oxidation of reduced dyes by fumarate. Originally they found the enzyme in crude preparations of the "old yellow enzyme" of Warburg & Christian (26). Thus far they have been unable to separate fumaric hydrogenase from other

flavoproteins in the "old yellow enzyme" preparation. The evidence favors FAD as the prosthetic group of fumaric hydrogenase. Since flavinmonophosphate is the prosthetic group of the Warburg & Christian "old yellow enzyme," there can be no doubt of the non-identity of fumaric hydrogenase and the "old yellow enzyme." In the process of isolating fumaric hydrogenase, the enzyme is partially split and FAD must be added to restore full activity. The identity of fumaric hydrogenase with eight of the known flavoproteins has been excluded by Fischer *et al.* There is a possibility that a highly purified flavoprotein isolated from top bakers' yeast by Green *et al.* in 1941 (27) may be identical with fumaric hydrogenase.

The search for antibacterial agents in molds has resulted in the isolation by Raistrick *et al.* and others (28 to 31) of a flavoprotein which has been identified with glucose oxidase. The final product obtained by Raistrick *et al.* appears to be homogeneous. Strains of *Penicillium notatum* W. secrete this enzyme into the growth medium. The prosthetic group of the enzyme is probably FAD (28). The enzyme catalyzes the aerobic oxidation of glucose to gluconic acid with the production of hydrogen peroxide. It is in fact the production of hydrogen peroxide which is at the root of the antibacterial action of the flavoprotein system (28). The addition of glucose to the enzyme bleaches the yellow color instantaneously. The reduced form of the enzyme in turn is oxidized by molecular oxygen. The enzyme thus undergoes a catalytic cycle of reduction by the substrate followed by oxidation of its leuco form in air with production of hydrogen peroxide.

Since the antibacterial action of glucose oxidase was presumed by Raistrick *et al.* to be due to hydrogen peroxide production it followed that other flavoprotein systems should have identical action. This was tested and found to be so in the case of milk xanthine oxidase (32, 33). Other flavoproteins will be more difficult to test since they are usually contaminated with catalase which nullifies the antibacterial effect.

Glycolysis and Fermentation

Cori and his co-workers have rounded off their researches on muscle phosphorylase with four elegant papers (34 to 37) on its preparation, properties, and kinetics. Under physiological conditions the enzyme is a firmly conjugated adenylic acid-protein compound which cannot be resolved by mild purification procedures such as salt pre-

cipitation. However, skeletal muscle contains an enzyme which splits off adenylic acid from phosphorylase. It is thus possible to obtain by enzymic means a split form of phosphorylase which is inactive unless supplemented with adenylic acid. Crystalline trypsin at pH 6 has the same effect as the muscle-splitting enzyme in resolving phosphorylase.

Phosphorylase from skeletal muscle has been obtained in highly purified and crystalline state though the best preparations are not completely homogeneous. According to Oncley (38) the molecular weight is probably about 340,000 to 400,000 assuming a value of 0.74 for partial specific volume. The amount of phosphorylase in skeletal muscle is rather high (40 to 80 mg. per 100 gm.) and is roughly half that of zymohexase.

It has been known for some time that the presence of certain polysaccharides like glycogen and amylopectin is necessary to catalyze the enzymic condensation of glucose-1-phosphate to polysaccharide. In other words, polysaccharide cannot be formed unless a highly branched polysaccharide is already present. Cori *et al.* interpret this effect as follows: The catalytic polysaccharides are in effect nuclei which grow by deposition of glucose units. For the sake of simplicity, we may regard the catalytic polysaccharides as consisting of a central core with large numbers of spurs coming off, each spur averaging from 6 to 18 glucose units in length. The glucose unit at the end of each spur condenses with glucose-1-phosphate by 1 : 4 linkage, and the process is continued until each spur, according to Hassid *et al.* (39), attains a straight chain length of some 200 glucose units. The net effect is the conversion of a highly branched catalytic polysaccharide into an essentially unbranched non-catalytic amylose-type polysaccharide. The iodine color serves as an indicator of the degree of branching. A pure blue color such as is given by amylose means zero or very slight branching. The greater the degree of branching the more the iodine color tends towards brown or red. Whereas phosphorylase from skeletal muscle yields a polysaccharide which stains iodine blue, the corresponding enzymes from liver and heart yield a glycogen-like product which stains iodine brown or brownish red. Cori *et al.* regard the synthesis of highly branched glycogen from glucose-1-phosphate as the result of the collaboration of two enzymes one of which catalyzes condensations of glucose-1-phosphate with terminal glucose by 1 : 4 linkage while the other presumably catalyzes condensation by 1 : 6 linkage.

Under standard conditions each molecule of phosphorylase con-

denses 4×10^4 molecules of glucose-1-phosphate per minute. Relatively high concentrations of a reducing agent like cysteine and a polysaccharide like glycogen must be present for maximum activity to be attained. When phosphorylase is split into its component parts adenosinemonophosphate but not adenosinediphosphate or adenosinetriphosphate restores activity. Inosinemonophosphate has a weak effect in high concentrations only.

Buchanan *et al.* (40) have made some studies with radioactive carbon on glycogen synthesis from acetic, propionic, and butyric acids. They found glycogen formation from the 3 and 4 carbon fatty acids but not from the 2 carbon fatty acid. Since they showed previously that pyruvic acid gives rise to glycogen (41) it now appears that both the 3 and 4 carbon fatty acids eventually give rise to pyruvic acid.

Doudoroff (42, 43) has obtained a cell free enzyme from *Pseudomonas saccharophilia* which catalyzes the phosphorylysis of sucrose to glucose-1-phosphate and fructose. There is some evidence that the reaction is reversible since sucrose can be formed when starting with mixtures of glucose-1-phosphate and fructose. No hexose catalyst is required for sucrose synthesis. The enzyme is specific for sucrose being without effect on trehalose, maltose, and glucose. Neither fructose-1-phosphate nor fructose diphosphate can replace fructose in the condensation reaction. Kagan *et al.* (44) have made similar observations on *Leuconastor mesenteroides*. Under the conditions of Kagan's experiments, fructose formed by phosphorylysis of sucrose disappears in some side reaction.

Warburg & Christian (45) have prepared zymohexase from rat muscle in highly purified and crystalline state but obtained no information about its prosthetic group or active groups. They claim that their enzyme from rat muscle is twice as active as the homogeneous enzyme isolated in 1940 from rabbit muscle by Herbert *et al.* (46). Since the methods and conditions for estimating activity as well as the sources of the enzyme were different in the two cases, the significance of the comparison is dubious.

Meyerhof & Junowicz-Kocholaty (47) have gone to considerable pains to determine whether *d*-3-glyceraldehyde phosphate actually combines with inorganic phosphate before oxidation takes place. Since the equilibrium reactions in which *d*-3-glyceraldehyde phosphate is involved were not affected by the concentration of inorganic phosphate, the authors were of the opinion that phosphate formed a loose physical addition product rather than a definite chemical compound.

Kalckar (48) has isolated in highly purified state myokinase, an enzyme found in skeletal muscle which catalyzes the reversible dismutation of adenosinediphosphate to adenosinemonophosphate and adenosinetriphosphate. Approximately 60 per cent of adenosinediphosphate is converted into the two products of dismutation at equilibrium. The enzyme can be heated in boiling 0.1 *N* hydrochloric acid and precipitated with trichloroacetic acid without loss of activity. It is apparently not identical with insulin. It is found in large amounts in skeletal muscle, in traces in heart and brain, and not at all in liver and kidney.

Kubowitz & Ott (49) have added another to the long list of glycolytic enzymes which they are preparing in highly purified state. They have now isolated the lactic acid dehydrogenating enzyme of rat sarcoma in the form of a crystalline mercury protein complex which can be converted into an active form by dialysis against hydrogen cyanide or cysteine. Straub in 1940 (50) was the first to report the preparation of lactic dehydrogenase in a homogeneous and crystalline state. He has also brought the malic dehydrogenase of pig heart to a similar high degree of purity (51). Both these enzymes require the presence of coenzyme I for activity. The activity per mg. dry weight is practically identical for the two enzymes ($Q_{O_2} =$ ca 60,000).

In 1939 Engelhardt & Lyubimova (52) reported that the adenosinetriphosphate activity of muscle was almost entirely associated with the myosin fraction and they suggested that the enzyme might be identical with myosin itself. D. M. Needham has confirmed their observation that myosin preparations split off one phosphate group from adenosine triphosphate but have little or no action on adenosinediphosphate (53). She has also shown that myosin while very active as adenosinetriphosphate has no phosphatase action on α-glycerophosphate or hexosediphosphate, nor can it catalyze the transfer of phosphate from adenosinetriphosphate to fructose-6-phosphate. Bailey (54) has studied the activation of myosin adenosinetriphosphatase by divalent metals, particularly calcium, magnesium, and manganese, and he has found that the myosin enzyme is unique among phosphatases in the way it is affected by divalent cations. He regards all the available evidence as consistent with the assumption that myosin and adenosinetriphosphatase are identical and suggests "that stimulation of muscle is connected with the availability of calcium ions to the myosin adenosinetriphosphatase fibrillar surface."

Ziff (55) has studied the action of pharmacological agents on myo-

sin adenosinetriphosphatase activity and found little or no effect. Copper and zinc salts were strongly inhibitory.

The mode of action of the sulfa drugs has been shown to be tied up inextricably with the resemblance between *p*-aminobenzoic acid and the sulfonamides. Since the role of *p*-aminobenzoic acid in enzyme reactions is obscure, this resemblance has been suggestive but not conclusive as far as establishing that the bacteriostatic effects of the sulfa drugs are explicable in terms of their effects on some enzyme system. In the case of gramicidin the parallelism of bacteriostasis and inhibition of some key enzyme system is now well established. Dubos, Coburn & Hotchkiss (56) have shown that gramicidin in concentrations which inhibit growth has a very pronounced inhibitory or stimulating effect on the respiration of susceptible bacteria. Cephalin which counteracts the bacteriostatic effect of gramicidin also counteracts the effects of gramicidin on respiration (57). More recently Hotchkiss (58) has analyzed the effect of gramicidin in some detail using extracts of animal cells in addition to bacterial suspensions for experimental material. His results indicate that gramicidin interferes with a mechanism which couples phosphorylation of glucose with certain oxidation processes. This mechanism is an essential one not in the fermentation or glycolysis of glucose, but probably in the utilization of the energy liberated during fermentation. Thus, in the presence of gramicidin, fermentation is speeded up, but the esterification of glucose which is coupled with the oxidation reaction is suppressed. Gramicidin may well provide a tool for the elucidation of some coupled phosphorylation oxidoreductions which hitherto have been wrapped in obscurity.

Furchgott & Shorr (59) have studied in great detail phosphate exchange in resting cardiac muscle by means of radioactive phosphate. Davydova (60) has studied the properties of the enzyme system which links phosphorylation of glucose with oxidation of succinic acid. This system requires the presence of adenine nucleotides. Oxidation can go on without phosphorylation but phosphorylation is impossible without oxidation.

Coenzymes and Prosthetic Groups

Gale & Epps (61) have discovered that lysine decarboxylase extracted from *B. cadaveris* can be split in alkaline solution and reconstituted on addition of a coenzyme which apparently is not identical with any of those described hitherto. The coenzyme which is widely

distributed in animal tissues and microorganisms has been prepared from brewers' yeast in a highly purified state. Thus 1μg. of coenzyme carbon catalyzed the evolution of 1800 μl. of carbon dioxide per hour from lysine in the presence of the split enzyme. The concentrated solution of the purified coenzyme was deep yellow but contained no flavin. It was stable in boiling 0.1 *N* sodium hydroxide but not in boiling 0.1 *N* sulfuric acid.

Braunshteĭn & Kritsman (62) have continued their purification of the coenzyme of aspartic transaminase. This coenzyme is different from all the known coenzymes though its relation to the coenzyme of the lysine decarboxylase has yet to be clarified. It has been prepared from extracts of pig heart. It is strongly basic, soluble in water, and insoluble in organic solvents. It forms insoluble salts with silver and mercury in alkaline solution. Little can yet be said about its chemical constitution except that phosphorus is not present.

Since *p*-aminobenzoic acid (pab) has been regarded as a possible essential metabolite or constituent of some enzyme system it may be of interest to mention here the isolation from yeast by Ratner, Coburn & Green of a peptide of "pab" (63). The peptide contains some thirteen molecules of *l*(+)-glutamic acid and one molecule of "pab." The amino group of "pab" is free. This peptide has no antisulfonamide action except after hydrolysis to free "pab."

Carboxylases

This group of enzymes has received particularly close attention in recent years. Gale following his work on the oxidation of amines by bacteria has extracted lysine carboxylase from acetone dried *B. cadaveris* (61). The enzyme specifically attacked *l*-lysine. The product of decarboxylation was the corresponding diamine, cadaverine ($NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2$). The activity of the purified carboxylase was formidable ($Q^{C}_{CO_2}$ of 46,000). The resolution of lysine carboxylase into a protein and a prosthetic group was effected by exposure to ammoniacal ammonium sulfate. The details of the chemical properties of the prosthetic group are given above.

Davies (64) has announced the preparation from *Cl. acetobutylicum* of an enzyme which catalyzes the decarboxylation of acetoacetic acid to acetone. The enzyme is quite stable both in the cell and in the aqueous extract. It has been obtained in a highly purified form. There are hints that it dissociates in dilute solutions but the evidence is as yet not conclusive. The enzyme specifically attacked acetoacetic acid.

Blaschko (65) has studied the properties of a cysteic acid ($SO_3H \cdot CH_2 \cdot CHNH_2 \cdot COOH$) carboxylase which he prepared from liver. The product of decarboxylation is taurine ($SO_3H \cdot CH_2 \cdot$-CH_2NH_2). Cysteic acid carboxylase has many resemblances to "dopa" carboxylase, the enzyme which catalyzes the decarboxylation of dihydroxyphenylalanine to the corresponding amine. Both carboxylases are cyanide sensitive and attack the natural forms of the amino acids exclusively. However the fact that these two enzymes do not always accompany one another excludes the possibility of their identity.

Kalnitsky & Werkman (66) have obtained a cell free extract of *E. coli* which catalyze the anaerobic decarboxylation of pyruvic acid. Since apparently more than one enzyme is involved the products of more than one reaction are found, viz. acetic, formic, lactic, and succinic acids. The carboxylase or carboxylases involved are inactivated by dialysis, but activity is restored on addition of boiled yeast juice. Inorganic phosphate, manganese, and co-carboxylase are some of the components of the system.

The enzyme which catalyzes the decarboxylation of oxaloacetic acid to pyruvic acid also catalyzes the reverse reaction. Thus carbon dioxide can combine with pyruvic acid to form oxaloacetic acid. Krampitz *et al.* (67) showed this very prettily with the use of radioactive carbon dioxide in the oxaloacetic acid-carboxylase system of bacteria. Evans *et al.* (68) have studied the properties of an enzyme from pig liver which catalyzes the reversible decarboxylation of oxaloacetic acid. The enzyme is activated by manganese ions. The fixation of carbon dioxide by pyruvic acid in the presence of oxaloacetic acid carboxylase probably accounts for most, if not all the results on the formation and fixation of carbon dioxide by animal tissues reported by Hastings *et al.* (69 to 72).

Ochoa (73) has analyzed the oxidative decarboxylation of α-ketoglutaric acid by crude extracts of cat heart. This process was found to be accompanied by phosphorylation of glucose. Diphosphothiamin and divalent metals have some effect on Ochoa's system.

Long (74) has further investigated the oxidation of pyruvate and α-ketoglutaric acid in ground preparations of pigeon brain. The system employed is very complex and involves more than one enzyme. Apparently inorganic phosphate and adenine nucleotide are required in addition to diphosphothiamin though they may catalyze different stages in the over-all reaction.

Rice & Evans (75) have undertaken an analysis of the stimu-

lating effect of insulin which Krebs & Eggleston (76) were the first to observe on the respiration of fresh suspensions of pigeon breast muscle. Their data suggest that insulin increases the ability of the brei to oxidize pyruvic acid aerobically. Since the oxidation of pyruvate is malonate sensitive, the authors suggest that insulin affects the action of citrogenase, the enzyme which catalyzes the condensation of pyruvic and oxaloacetic acids to form some precursor of citric acid.

Acetylation of Amines

With the discovery by Lipmann (77) that the enzymes which oxidize pyruvic acid in various bacteria form acetylphosphate, it has been suggested that acetylphosphate is probably the acetylating agent of animal cells. To date, however, acetylphosphate has not been identified as a product of the oxidation of pyruvic acid in animal tissues. Furthermore there is now a body of evidence in favor of acetic acid as an acetylating agent. Thus Bernhard & Steinhauser (78) showed that when γ-phenylaminobutyric acid was fed to rats and dogs with simultaneous administration of deuterioacetic acid or deuterioethylalcohol, deuterio acetyl γ-phenylaminobutyric acid could be isolated from the urine. They concluded that acetic acid as such was responsible for the acetylation. Bloch & Rittenberg (79) have confirmed and extended this observation. Klein & Harris (80) in 1938 studied the acetylation process in tissue slices. They found that the process was confined to liver and that acetic acid was apparently more effective than any other substance in acetylating sulfanilamide. The enzyme system concerned did not survive the disintegration of the cell.

Doisy & Westerfeld (81) studying the acetylation of *p*-aminobenzoic acid by rabbits observed that administration of acetoin and of 2,3-butylene glycol significantly increased the degree of acetylation over that of the control whereas sodium acetate was without any positive effect. In contrast, Martin & Rennebaum (82) found that acetylation of sulfanilamide was greatly increased by pyruvate. This effect surpassed greatly that of acetoin. They also failed to find any increase in the degree of acetylation over the control when acetate was fed.

There is one important consideration which has some bearing on attempts to influence cellular reactions by dietary feeding. If these reactions occur normally, as is the case apparently for acetylation of

amines, then the limiting factor may not be the concentration of the substrate. In other words, the enzymes are already working at maximal velocity. Further addition of substrate in the diet will exert no appreciable influence under these conditions. Negative results therefore may have no bearing on the question of whether the particular substance fed intervenes in the acetylation process.

Nachmansohn *et al.* (83, 84) have been analyzing the enzyme system which anaerobically acetylates choline. The choline acetylating system depends upon the presence of adenosinetriphosphate. Purification of the system is complicated by the fact that more than one enzyme is involved. When the system is dialyzed, activity is lost, but addition of certain amino acids particularly glutamic acid restores the original activity. Succinic acid and citric acid are also quite active. The significance of these activations is not yet clear.

Citric Acid Cycle

The citric acid cycle proposed by Krebs (85, 86, 87) to provide a mechanism for the complete breakdown of pyruvic acid to carbon dioxide and water has been a stormy petrel in recent years. It is not proposed to discuss here to what extent the citric acid cycle does or does not account for what is referred to as a carbohydrate oxidation in animal tissues. By and large such discussions are not very fruitful in the present state of our knowledge. However, some very exciting contributions have been made to our knowledge of the enzymic mechanisms for the synthesis of citric acid. Breusch (88) was the first to announce the discovery in kidney of citrogenase, an enzyme which catalyzed the condensation of pyruvic and oxaloacetic acids to form citric acid or some precursor thereof. Wieland & Rosenthal (89) have followed up this original observation of Breusch with a delightful analysis of the mechanism of citric acid formation in kidney and heart brei. They found that mixtures of oxaloacetic and acetoacetic acids rapidly give rise to citric acid in the presence of kidney brei and barium ions. The function of the barium ions is to inhibit the breakdown of citric acid once it has been formed. Pyruvic acid may replace acetoacetic acid but it has only 30 to 50 per cent of the latter's activity. Although no oxidation is supposed to be involved, nevertheless the condensation requires the presence of oxygen and is accelerated in the presence of pure oxygen as compared to air. Wieland & Rosenthal postulate that oxaloacetic and acetoacetic acids condense to form either citroylacetic acid or acetyl citric acid, both of which yield citric

and acetic acids on hydrolysis. Since acetic acid was not found as an end product together with citric acid the authors suggest that one or the other of the above procitric acids condenses with another molecule of oxaloacetic acid to form a compound which can yield two molecules of citric acid on hydrolysis but without simultaneous liberation of acetic acid.

$$HO_2{\cdot}C{\cdot}CH_2{\cdot}CO{\cdot}COOH + HO_2{\cdot}C{\cdot}CH_2{\cdot}CO{\cdot}CH_3 \rightarrow \begin{cases} \underset{\text{citroylacetic acid}}{HO_2C{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH_2CO{\cdot}CH_2{\cdot}CO_2H} \\ \underset{\text{acetylcitric acid}}{HO_2C{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH(CO_2H){\cdot}CO{\cdot}CH_3} \end{cases}$$

$$HO_2C{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH_2{\cdot}CO{\cdot}CH_2CO_2H + HO_2C{\cdot}CH_2{\cdot}CO{\cdot}CO_2H \rightarrow$$

$$HO_2C{\cdot}CH_2{\cdot}C(OH)(CO_2H){\cdot}CH_2{\cdot}CO{\cdot}CH(CO_2H){\cdot}C(OH)(CO_2H){\cdot}CH_2{\cdot}CO_2H$$

The citric acid forming enzyme is found in kidney and heart but not in liver. To explain why pyruvic acid can replace acetoacetic acid in the condensation reaction they postulate the Krebs' mechanism (90) by which pyruvic acid can condense with its oxidation product to form acetopyruvic acid which in turn yields acetoacetic acid on hydrolysis.

Breusch (91) has extended his original observations on citrogenase, and independently of Wieland & Rosenthal he has found evidence for the following general reaction:

$$\underset{\beta\text{-keto acid}}{RCO \cdot CH_2 \cdot COOH} + \underset{\text{oxaloacetic acid}}{COOH \cdot CO \cdot CH_2 \cdot COOH} \rightarrow$$

$$R \cdot COOH + \underset{\text{citric acid}}{COOH \cdot CH_2 \cdot COH\,(\cdot COOH) \cdot CH_2 \cdot COOH}$$

Some eight β-keto acids were found which could replace acetoacetic acid in this reaction including β-keto dicarboxylic acids. Breusch does not set forth the experimental evidence for the formation of RCOOH in his preliminary note. It would be interesting to know how he accounts for the fact that no acetic acid accumulates when acetoacetic acid condenses with oxaloacetic acid.

Lynen (92) has demonstrated unequivocally that the individual steps in the citric acid cycle can take place in the yeast cell. He showed for the first time that yeast contains a typical succinic acid dehydrogenase which like that of animal tissues is inhibited by malonate. Apparently succinic acid does not penetrate the yeast cell at neutral pH,

but does when the external pH is lowered to 1 or 2. The same applies to malonic acid. According to Lynen acetic acid condenses with some aldehyde ($R \cdot CH_2 \cdot CHO$) to form $CH_3 \cdot CO \cdot CH(\cdot R) \cdot CHO$, which in turn condenses with oxaloacetic acid to form procitric acid. On hydrolysis the citric acid precursor yields citric acid and the original aldehyde. Citric acid then undergoes the same fate as in animal tissues. In other words the citric acid cycle in yeast provides a mechanism for the complete oxidation of acetic acid whereas the cycle in certain animal tissues leads to the complete oxidation of acetoacetic acid or pyruvic acid. The cycle in both yeast and animal tissues leads to the regeneration of oxaloacetic acid. Citric acid yields oxaloacetic acid via α-ketoglutaric acid, succinic semi-aldehyde, succinic acid, fumaric acid, and malic acid as intermediaries. Since the oxidation of succinic acid to fumaric acid is inhibited by malonic acid, the cycle as a whole is inhibited by malonic acid.

Oxidation of Natural Amino Acids

Some progress has been made in the systematic study of the enzymes which catalyze the oxidation of natural amino acids. Ratner *et al.* (22) have discovered in kidney and liver an enzyme which catalyzes the oxidation of glycine to glyoxylic acid and ammonia, and of N-methyl glycine to glyoxylic acid and methylamine. No other natural amino acid is attacked by the enzyme. Substitution products of glycine and glycine peptides are also inactive. Since glyoxylic acid can be readily converted to oxalic acid by systems in liver and kidney, the origin from glycine of some if not all urinary oxalic acid is strongly indicated.

Ratner *et al.* (93) have discovered in rat kidney and liver an enzyme or group of enzymes which catalyzes the oxidative deamination of thirteen natural amino acids, viz., leucine, proline, norleucine, norvaline, methionine, valine, alanine, isoleucine, cystine, histidine, phenylalanine, tryptophane, and tyrosine. The corresponding keto acids are formed in each case. For each mole of amino acid oxidized, one mole of oxygen is taken up with production of one mole of hydrogen peroxide. N-methyl leucine and S-benzyl- or S-ethylcysteine are attacked more rapidly than any of the natural amino acids. Members of the dicarboxylic amino acids, diamino acids, and β-hydroxyamino acids are not attacked. Glycine also is inactive. This *l*-amino acid oxidase is specific for the *l* forms of the amino acids attacked. The method of preparation of the enzyme effectively eliminates all

traces of the *d*-amino acid oxidase. There is no evidence to substantiate the theory that the *d* enzyme is a degenerate form of the *l* (94). The assumption that enzymes change their specificity as a result of isolation procedures has been invoked and is still being invoked without there being a single authenticated case of such a transformation. Perhaps it may not be amiss to inquire into the experimental basis for these so-called enzyme artifacts.

The *l*-amino acid oxidase of rat kidney and liver is obtained in soluble form and can be purified by the usual procedures of protein fractionation. It is not inhibited either by hydrogen cyanide or capryl alcohol and in fact does not correspond in any way with the properties attributed to it on the basis of tissue slice experiments.

Stumpf & Green (95) have isolated from *Proteus vulgaris* what is unquestionably a single enzyme which catalyzes the oxidative deamination of some ten natural amino acids, viz., leucine, phenylalanine, histidine, arginine, norleucine, norvaline, methionine, tyrosine, tryptophane, and isoleucine. The corresponding keto acid is formed in each case. Unlike the animal enzyme it is partially cyanide sensitive and is completely inhibited by capryl alcohol. Furthermore it is associated with particles which can be readily sedimented in a centrifugal field of 100,000 x g. For each molecule of amino acid oxidized only one atom of oxygen is taken up and no hydrogen peroxide is formed. The *Proteus* enzyme unlike the animal enzyme does not oxidize N-methyl amino acids, proline, alanine, and valine, but does oxidize arginine.

Lang & Westphal (96) have evidence of the existence in rat liver of an enzyme which catalyzes the aerobic oxidation of *l*-phenylalanine and *l*-tyrosine. The products have not been identified, but there was no production of ammonia. Very likely oxidation took place in the ring.

Serine deaminase which was first studied by Gale & Stephenson (97) in *E. coli* is now being further investigated. Chargaff & Sprinson (98) have shown that the bacterial enzyme catalyzes the anaerobic conversion of serine to pyruvic acid and of threonine to α-keto butyric acid. Apparently both the *d* and *l* form of serine are attacked by the enzyme. Serine derivatives are not acted upon. Binkley (99) regards serine deaminase, cysteine desulfurase, and phosphoglyceric enolase as very similar if not identical. Thus they are all inactivated by dialysis and in each case activity is restored by addition of zinc, magnesium, or manganese ions. Each of the three enzymes is inhibited by the substrates of the other two and by 0.001 *M* fluoride. The three ac-

tivities accompany one another whether the extracts are prepared from microorganisms or mammalian tissue. The nature of the catalyses is very similar in each case but, whereas aminoacrylic acid is unstable and rapidly hydrolyzes to pyruvic acid and ammonia, enol phosphopyruvic acid is relatively stable and does not undergo further change.

a) $CH_2 \cdot OH \cdot CH \cdot NH_2 \cdot COOH \rightarrow [CH_2 : C \cdot NH_2 \cdot COOH] \rightarrow$
$[CH_3 \cdot C : NH \cdot COOH] \rightarrow CH_3 \cdot CO \cdot COOH + NH_3$

b) $CH_2 \cdot SH \cdot CH \cdot NH_2 \cdot COOH \rightarrow [CH_2 : C \cdot NH_2 \cdot COOH] + H_2S \rightarrow$
$[CH_3 \cdot C : NH \cdot COOH] \rightarrow CH_3CO \cdot COOH + NH_3$

c) $CH_2 \cdot OH \cdot CHOPO_3H_2 \cdot COOH \rightarrow CH_2 : COPO_3H_2 \cdot COOH$

Lipoxidase.—Sumner (100, 101), continuing his investigations on soy bean lipoxidase, has concluded that the enzyme catalyzes specifically the peroxidation of unsaturated fatty acids at the 9,10-double bond of cis configuration. Allyl alcohol, mesityl oxide, elaidic acid, and erucic acid were completely inactive. Oleic and eleostearic acids were peroxidized to the extent of 16 per cent of the theory for one double bond, while linoleic and linolenic acids were respectively peroxidized to the extent of 90 and 95 per cent. Balls *et al.* (103) tested the action of lipoxidase on exceptionally pure specimens of fats and fatty acids, and found that out of a considerable number only linoleic, linolenic, and arachidonic acids or their ethyl esters were attacked. Pure oleic acid was untouched. They postulated that lipoxidase specifically peroxidized biologically essential unsaturated fatty acids.

It has been known that carotene is not oxidized by lipoxidase unless an unsaturated fatty acid is present. Sumner (102) in analyzing this induced reaction has demonstrated that rapid oxidation of carotene is not brought about by action of fatty acid peroxides either directly or in the presence of lipoxidase. Induced oxidation of carotene occurs only when the process of enzymic peroxidization is actively taking place. Sumner concludes that carotene is oxidized by some intermediary oxide which is produced during peroxidation of the unsaturated fatty acid. This conclusion fits in with the observation that fat peroxidation is diminished coincidentally with the coupled oxidation of carotene.

Balls *et al.* (103) isolated from soy bean meal as well as from other plant and animal sources a polypeptide-like substance which greatly increased the activity of purified soybean lipoxidase. The protein-like nature of the activator was further demonstrated by its destruction following incubation with proteolytic enzymes like papain. The avail-

able evidence was consistent with the assumption that the activator is a protective agent for the enzyme and not a coenzyme.

Fatty acid dehydrogenase.—Adipose tissue according to Shapiro & Wertheimer (104) contains an enzyme which dehydrogenates the higher members of the fatty acids like stearic and palmitic acids in the presence of adenylic acid and inorganic phosphate. Liver, muscle, heart, and testis also contain the enzyme. With diminishing chain length of the fatty acid the activity of the enzyme decreases, approaching zero for valeric acid. Neutral fats are inactive while phospholipids and succinic acids are rapidly attacked. The usual inhibitors of glycolysis have no effect on the activity of this enzyme. Several years ago Lang & Mayer (105, 106) described a fatty acid dehydrogenase prepared from liver which very likely is identical with that of Shapiro & Wertheimer since the specificity and properties of the two enzymes correspond closely. Moreover, in both cases adenylic acid is reported to be the coenzyme. Oxygen rapidly inhibits the activity of the enzyme from adipose tissue. All activity measurements therefore were carried out anaerobically with methylene blue as hydrogen acceptor.

Muñoz & Leloir (107) have described the preparation of a labile enzyme from guinea pig liver which catalyzes the dehydrogenation of the lower fatty acids (3–8 carbon atoms). For maximum activity fumarate, cytochrome-*c*, inorganic phosphate, adenylic acid, and magnesium or manganese ions were required. Saturated fatty acids containing less than nine carbon atoms and crotonic acid, isocrotonic acid, and β-hydroxybutyric acid were attacked. Fluoride, iodoacetate, arsenate, and malonate completely inhibited the action of this enzyme. Methylene blue did not accelerate the aerobic oxidation.

Aldohexose dehydrogenase.—A labile aldohexose dehydrogenase has been isolated both from the seeds of *Phaseolus mungo* and from small intestine which dehydrogenates glucose, galactose, or mannose to ascorbic acid either anaerobically or aerobically [Rudra (108)]. Fructose and sorbose are converted to a small extent while disaccharides and pentoses are quite inactive. Manganese is apparently a component of the enzyme system. The reaction is sensitive to cyanide.

Uricase.—Scheer & Scheer (109) have worked out a simplified method for the preparation of highly purified uricase from pig liver. They have also shown that the addition of cysteine increases the rate of oxygen uptake of the uric acid-uricase system. There was no interaction of uric acid and cysteine since both reactants were found to be oxidized to their usual oxidation products, viz., allantoin and cystine,

respectively. Cysteine in absence of uric acid took up oxygen slowly in presence of the enzyme. The mechanism of this coupled oxidation of cysteine and uric acid in presence of uricase is obscure.

Succinoxidase.—Straub (110) examined the components of succinoxidase and confirmed a previous report by Stern & Melnick (111) that some factor is necessary for the reaction of succinic dehydrogenase with cytochrome-*c*. From pig heart muscle he obtained a preparation of succinic dehydrogenase which contained among other enzymes cytochrome oxidase and cytochromes-*a*, *b*, and *c*. This preparation was quite inactive with succinic acid. When he added, however, to this inactive preparation the SC Factor (succinic dehydrogenase-cytochrome binding factor), which he obtained from pig heart muscle and which did not contain succinic dehydrogenase, succinic acid was rapidly oxidized. Spectroscopic analyses of the rate of reduction of the different cytochromes further revealed that the SC Factor was an intermediary link in the reaction of succinic dehydrogenase with the cytochromes. Straub postulates the following series of reactions in which the arrows indicate the direction of transfer of hydrogen or electrons: succinic dehydrogenase → SC Factor → cytochrome-*c* → cytochrome-*a* → cytochrome oxidase. The SC Factor was prepared by heating a suspension of pig heart muscle at pH 9.2 and 55° for 15 minutes and neutralizing the cooled extract. Above pH 10 or below pH 4, it was rapidly destroyed.

In experiments with malic acid dehydrogenase, the SC Factor was quite inactive as an intermediate in the reaction between diaphorase and cytochrome-*c*.

Oxidation of glyoxylic acid and succinic semialdehyde.—The formation and oxidation of aldehyde acids has come to the fore in recent years. Ratner *et al.* (22) found that glyoxylic acid which is formed by oxidation of glycine gives rise to oxalic acid in presence of enzymes from liver, kidney, and muscle. Liver xanthine oxidase is particularly active in this respect. But in addition to the known aldehyde oxidizing enzymes there are hitherto undescribed enzymes in kidney and muscle which catalyze the oxidation of glyoxylic acid.

The formation of succinic semi-aldehyde by decarboxylation of α-ketoglutaric acid is catalyzed by an enzyme widely distributed in animal tissues [Westerfeld *et al.* (112)]. The further oxidation of succinic semi-aldehyde to succinic acid is rapidly catalyzed by several enzymes among them xanthine oxidase, liver aldehyde oxidase, and hitherto undescribed enzymes in kidney and muscle (113).

LITERATURE CITED

1. Agner, K., *Advances in Enzymology*, **3**, 137–48 (1943)
2. Theorell, H., and Åkeson, Å., *Arkiv Kemi, Mineral. Geol.*, **17**, no. 7, 1–6 (1943)
3. Theorell, H., *Enzymologia*, **10**, 250–52 (1942)
4. Keilin, D., and Mann, T., *Proc. Roy. Soc. (London) B*, **122**, 119–33 (1942)
5. Abrams, R., Altschul, A. M., and Hogness, T. R., *J. Biol. Chem.*, **142**, 303–16 (1942)
6. Sumner, J. B., and Gjessing, E. C., *Arch. Biochem.*, **2**, 295–99 (1943)
7. Theorell, H., Bergström, S., and Åkeson, Å., *Arkiv Kemi, Mineral. Geol.*, **16**, no. 13, 1–8 (1942)
8. Sumner, J. B., and Gjessing, E. C., *Arch. Biochem.*, **1**, 1–8 (1942)
9. Chance, B., *J. Cellular Comp. Physiol.*, **22**, 33–41 (1943)
10. Chance, B., *J. Biol. Chem.*, **151**, 553–77 (1943)
11. Agner, K., *Arkiv Kemi, Mineral. Geol.*, **16**, no. 6, 1–21 (1942)
12. Lemberg, R., and Legge, J. W., *Biochem. J.*, **37**, 117–27 (1943)
13. Agner, K., *Arkiv Kemi, Mineral. Geol.*, **17**, no. 9, 1–10 (1943)
14. Theorell, H., and Agner, K., *Arkiv Kemi, Mineral. Geol.*, **16**, no. 7, 1–14 (1942)
15. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. (London) B*, **119**, 114–40 (1936)
16. Keilin, D., and Hartree, E. F., *Nature*, **152**, 626 (1943)
17. Haas, E., *J. Biol. Chem.*, **148**, 481–93 (1943)
18. Granick, S. and Michaelis, L., *J. Biol. Chem.*, **147**, 91–97 (1943)
19. Michaelis, L., Coryell, C. C., and Granick, S., *J. Biol. Chem.*, **148**, 463–80 (1943)
20. Granick, S., *J. Biol. Chem.*, **149**, 157–67 (1943)
21. Hahn, P. F., Granick, S., Bale, W. F., and Michaelis, L., *J. Biol. Chem.*, **150**, 407–12 (1943)
22. Ratner, S., Nocito, V., and Green, D. E., *J. Biol. Chem.*, **152**, 49–133 (1944)
23. Warburg, O., and Christian, W., *Biochem. Z.*, **295**, 261 (1938)
24. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150–68 (1938)
25. Fischer, F. G., Roedig, A., and Rauch, K., *Ann.*, **552**, 203–42 (1942)
26. Warburg, O., and Christian, W., *Biochem. Z.*, **254**, 438–58 (1932)
27. Green, D. E., Knox, W. E., and Stumpf, P. K., *J. Biol. Chem.*, **138**, 775–80 (1941)
28. Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H., *Nature*, **150**, 634–35 (1942)
29. Van Bruggen, J. R., Reithel, F. J., Cain, C. K., Katzman, P. A., Doisy, E. A., Muir, R. D., Roberts, E. C., Gaby, W. L., Homan, D. M., and Jones, L. R., *J. Biol. Chem.*, **148**, 365–78 (1943)
30. Kocholaty, W., *Arch. Biochem.*, **2**, 73–86 (1943)
31. Schales, O., *Arch. Biochem.*, **2**, 487–90 (1943)
32. Lipmann, F., and Owen, C. R., *Science*, **98**, 246–48 (1943)

33. GREEN, D. E., AND PAULI, R., *Proc. Soc. Exptl. Biol. Med.*, **54**, 148–50 (1943)
34. GREEN, A. A., AND CORI, G. T., *J. Biol. Chem.*, **151**, 21–29 (1943)
35. CORI, G. T., AND GREEN, A. A., *J. Biol. Chem.*, **151**, 31–38 (1943)
36. CORI, C. F., CORI, G. T., AND GREEN, A. A., *J. Biol Chem.*, **151**, 39–55 (1943)
37. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.*, **151**, 57–63 (1943)
38. ONCLEY, J. L., *J. Biol. Chem.*, **151**, 27–28 (1943)
39. HASSID, W. Z., CORI, G. T., AND MACREADY, R. M., *J. Biol. Chem.*, **148**, 89–96 (1943)
40. BUCHANAN, J. M., HASTINGS, A. B., AND NESBETT, F. B., *J. Biol. Chem.*, **150**, 413–25 (1943)
41. BUCHANAN, J. M., HASTINGS, A. B., AND NESBETT, F. B., *J. Biol. Chem.*, **145**, 715–16 (1942)
42. DOUDOROFF, M., *J. Biol. Chem.*, **151**, 351–61 (1943)
43. DOUDOROFF, M., KAPLAN, N., AND HASSID, W. Z., *J. Biol. Chem.*, **148**, 67–75 (1943)
44. KAGAN, B. O., LYATKER, S. N., AND TSVASMAN, E. M., *Biokhimiya*, **7**, 93–108 (1942)
45. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **314**, 149–76 (1943)
46. HERBERT, D., GORDON, H., SUBRAHMANYAN, V., AND GREEN, D. E., *Biochem. J.*, **34**, 1108–23 (1940)
47. MEYERHOF, O., AND JUNOWICZ-KOCHOLATY, R., *J. Biol. Chem.*, **149**, 71–92 (1943)
48. KALCKAR, N. M., *J. Biol. Chem.*, **148**, 127–37 (1943)
49. KUBOWITZ, F., AND OTT, P., *Biochem. Z.*, **314**, 94–117 (1943)
50. STRAUB, F. B., *Biochem. J.*, **34**, 483–86 (1940)
51. STRAUB, F. B., *Z. physiol. Chem.*, **275**, 63–72 (1942)
52. ENGELHARDT, V. A., AND LYUBIMOVA, M. N., *Nature*, **144**, 668–69 (1939)
53. NEEDHAM, D. M., *Biochem. J.*, **36**, 113–20 (1942)
54. BAILEY, K., *Biochem. J.*, **36**, 121–39 (1942)
55. ZIFF, M., *Proc. Soc. Exptl. Biol. Med.*, **51**, 249–51 (1942)
56. DUBOS, R. J., HOTCHKISS, R. D., AND COBURN, A. F., *J. Biol. Chem.*, **146**, 421–26 (1942)
57. DUBOS, R. J., COBURN, A. F., AND HOTCHKISS, R. D. (Unpublished observations)
58. HOTCHKISS, R. D. (Unpublished observations)
59. FURCHGOTT, R. F., AND SHORR, E., *J. Biol. Chem.*, **151**, 65–86 (1943)
60. DAVYDOVA, S. Y., *Biokhimiya*, **7**, 13–24 (1942)
61. GALE, E., AND EPPS, H., *Nature*, **152**, 327–28 (1943)
62. BRAUNSHTEĬN, A. E., AND KRITSMAN, M. G., *Biokhimiya*, **8**, 1–8 (1943)
63. RATNER, S., COBURN, A. F., AND GREEN, D. E. (Unpublished observations)
64. DAVIES, R., *Biochem. J.*, **37**, 230–38 (1943)
65. BLASCHKO, H., *Biochem. J.*, **36**, 571–74 (1942)
66. KALNITSKY, G., AND WERKMAN, C. H., *Arch. Biochem.*, **2**, 113–24 (1943)
67. KRAMPITZ, L. O., WOOD, H. S., AND WERKMAN, C. H., *J. Biol. Chem.*, **147**, 243–55 (1943)

68. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771–84 (1943)
69. Conant, J. B., Cramer, R. D., Hastings, A. B., Klemperer, F. W., Solomon, A. K., and Vennesland, B., *J. Biol. Chem.*, **137**, 557–66 (1941)
70. Vennesland, B., Solomon, A. K., Buchanan, J. M., Cramer, R. D., and Hastings, A. B., *J. Biol. Chem.*, **142**, 371–77 (1942)
71. Vennesland, B., Solomon, A. K., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **142**, 379–86 (1942)
72. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171–82 (1941)
73. Ochoa, S., *J. Biol. Chem.*, **149**, 577–78 (1943)
74. Long, C., *Biochem. J.*, **37**, 215–25 (1943)
75. Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470–71 (1943)
76. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **32**, 913–25 (1938)
77. Lipmann, F., *Advances in Enzymology*, **1**, 99–162 (1941)
78. Bernhard, K., and Steinhauser, H., *Z. physiol. Chem.*, **273**, 31–38 (1942)
79. Bloch, K., and Rittenberg, D. (Unpublished observations)
80. Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, **124**, 613–26 (1938)
81. Doisy, E. A., Jr., and Westerfeld, W. W., *J. Biol. Chem.*, **149**, 229–36 (1943)
82. Martin, G. J., and Rennebaum, E. H., *J. Biol. Chem.*, **151**, 417–26 (1943)
83. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397–404 (1943)
84. Nachmansohn, D., John, H. M., and Waelsch, H., *J. Biol. Chem.*, **150**, 485–86 (1943)
85. Krebs, H. A., and Johnson, W. A., *Enzymologia*, **4**, 148–56 (1937)
86. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **34**, 442–59 (1940)
87. Krebs, H. A., *Biochem. J.*, **34**, 460–63 (1940)
88. Breusch, F. L., *Biochem. J.*, **33**, 1757–70 (1939)
89. Wieland, H., and Rosenthal, C., *Ann.*, **554**, 241–60 (1943)
90. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 772–79 (1937)
91. Breusch, F. L., *Science*, **97**, 490–92 (1943)
92. Lynen, F., *Ann.*, **554**, 40–68 (1943)
93. Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **148**, 461–62 (1943)
94. Krebs, H. A., *Biochem. J.*, **29**, 1620–44 (1935)
95. Stumpf, P. K., and Green, D. E., *J. Biol. Chem.* (In press)
96. Lang, K., and Westphal, U., *Z. physiol. Chem.*, **276**, 179–90 (1942)
97. Gale, E., and Stephenson, M., *Biochem. J.*, **32**, 392–404 (1938)
98. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **148**, 249–50 (1943)
99. Binkley, F., *J. Biol. Chem.*, **150**, 261–62 (1943)
100. Sumner, R. J., *J. Biol. Chem.*, **146**, 211–13 (1942)
101. Sumner, R. J., and Tessler, D. K., *Ind. Eng. Chem.*, **35**, 921 (1943)
102. Sumner, R. J., *J. Biol. Chem.*, **146**, 215–18 (1942)
103. Balls, A. K., Axelrod, B., and Kies, M. W., *J. Biol. Chem.*, **149**, 491–504 (1943)

104. Shapiro, B., and Wertheimer, E., *Biochem. J.*, **37**, 102–4 (1943)
105. Lang, K., and Mayer, H., *Z. physiol. Chem.*, **261**, 249–52 (1939)
106. Lang, K., and Mayer, H., *Z. physiol. Chem.*, **262**, 120–22 (1939)
107. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **147**, 355–62 (1943)
108. Rudra, M. M., *Nature*, **151**, 641–42 (1943)
109. Scheer, B. T., and Scheer, M. A. R., *J. Biol. Chem.*, **150**, 359–61 (1943)
110. Straub, F. B., *Z. physiol. Chem.*, **272**, 219–26 (1942)
111. Stern, K. G., and Melnick, J. L., *Nature*, **144**, 330 (1939)
112. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69–84 (1942)
113. Nocito, V., Ratner, S., and Green, D. E. (Unpublished observations)

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NON-OXIDATIVE ENZYMES

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Recent research on enzymes continued along several lines. Considerable effort was directed towards the purification of enzymes but opinions are still divided as to what constitutes the best criterium of enzyme purity. Taking into account the fact that an enzyme is distinct from other proteins owing to its highly specific catalytic ability, it would seem that the purification procedure should aim first of all at the achievement of the highest degree of activity. Occasionally, too much weight is attached to physicochemical criteria such as, for example, crystallization. From what we know of certain enzymes, it is fairly obvious that their catalytic activity may be adversely affected by the crystallization procedure. A crystalline enzyme preparation may contain a comparatively high percentage of impurities, whereas negligible amounts of mucilagenous admixture in an otherwise pure preparation may seriously impede the crystallization ability (1, 2).

Much progress has been made in the elucidation of the chemical nature of the active or prosthetic groups of several enzymes. In some instances the evidence for the existence of such a group was satisfactorily established on the basis of proportionality between the activity and the content, in the enzyme preparations, of the prosthetic group. On the other hand, several inorganic ions were claimed as essential components of enzymes merely because their addition stimulated the enzymic activity. It may be argued of course, that some of these ions occur in fact in the tissues and that they act by forming "dissociable complexes" with the enzyme proteins rather than as prosthetic groups proper. However, care should be exercised before the existence of such complexes is pronounced. Laccase was regarded for many years as a manganese-protein compound simply because manganese salts stimulated the laccase activity and were present in crude enzyme preparations. As a matter of fact, however, purified laccase is a manganese-free copper-protein and neither manganese nor copper salts have any effect whatever on the pure enzyme (3). Carbonic anhydrase, on the other hand, is inhibited by several inorganic ions, zinc among others, and yet zinc forms the prosthetic group

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of the enzyme (4). Similar remarks apply to claims made with regard to the chemical nature of the prosthetic groups, if they are based solely on the effect of inhibitors. It is perfectly reasonable to assume that the instantaneous and reversible inhibition of an enzyme by metal-binding substances such as cyanide, azide, or hydrogen sulphide usually indicates that the enzyme in question contains a metal. However, it is not equally justifiable to regard the inability of metal-binding substances to produce inhibition as a proof that a metal does not constitute a part of the enzyme molecule. Thus, for example, $\alpha\alpha'$-dipirydyl has no effect on catalase or peroxidase yet both of them contain iron; or 8-hydroxyquinoline may have hardly any influence on phenol oxidases which are copper-containing enzymes.

Much interesting work has been done on the mechanism of action of certain inhibitors (5, 6, 7). In addition, specific enzyme inhibitors have been successfully employed in physiological studies of the enzyme function within intact cells and organisms, particularly so with choline esterase and carbonic anhydrase. Inhibitors were also used in investigations concerned with the site of enzyme formation in the body (8).

Purified enzymes also served as delicate tools in the unraveling of the chemical structure of various complex substances such as lipids (9), nucleic acids (10, 11), and certain carbohydrates (12). Enzymes are becoming increasingly popular in the quantitative evaluation of vitamins and other substances (13, 14, 15). Furthermore, enzymes are applied on an ever increasing scale in the search for active groups or linkages in biologically potent substances such as hormones and viruses (16, 17). An encouraging and promising aspect of enzyme study is found in the successful practical application of enzyme research to medical problems (diffusing factors; renin; study of chemotherapeutic agents in relation to various enzymes; etc.).

Enzymes Engaged in Carbohydrate Metabolism

Considerable progress has been made during the last few years in the research on enzymes which control the intermediary reactions of glycogenolysis and of alcoholic fermentation. Among these enzymes two groups predominate, the hydrogen transferring enzymes or dehydrogenases and the phosphate transferring enzymes or phosphopherases. The scheme on page 30 demonstrates the position of these and other enzymes in the anaerobic breakdown of glycogen to lactic acid (muscle) and of glucose to alcohol and carbon

dioxide (yeast). There are indications that apart from glycogen and glucose, other sugars as well can be metabolized in an analogous manner. According to Kosterlitz (18) such a possibility exists for galactose. He assumes that during the adaptation of yeast to ferment galactose, two new enzymes are formed, one which phosphorylates galactose at C_1 and another, which converts 1-phosphogalactose to 6-phosphoglucose, probably by way of 1-phosphoglucose. The phosphorolysis of sucrose was observed in cultures of *Leuconostoc mesenterioides* (19) and in dried cell preparations of *Pseudomonas saccharophila* (20). In both instances the ester formed was identified as 1-phosphoglucose. A mechanism similar to that of glucose fermentation probably operates also in the fermentation of maltose by yeast (21).

Phosphorylase.—The formation of the first product of glycogenolysis, the Cori ester or $d(+)$1-phospho-α-glucopyranose, is brought about by an enzymic disruption of the polysaccharide, a process for which Parnas coined the name "phosphorolysis." The phosphorolysis is a reversible reaction between inorganic phosphate and glycogen or starch, catalyzed by the enzyme phosphorylase. The equilibrium state defined by the values of the ratio inorganic phosphorus/ester phosphorus, is unaffected by wide variations in the concentration of the polysaccharide present. On the other hand, it is markedly influenced by alterations in the concentrations of hydrogen ions; as the pH values increase from 5 to 7, the values of the ratio free phosphorus to esterified phosphorus decrease from about 11 to 3 (22). Pure muscle phosphorylase has been isolated by Green, Cori & Cori (23) from the aqueous extract of rabbit skeletal muscle where it constitutes 2 per cent of the total extractable protein. The enzyme requires adenylic acid for the disruption of glycogen and has been obtained as a crystalline complex with adenylic acid. The resynthesis of polysaccharide by phosphorylase requires also the addition of traces of glycogen. However, the polysaccharide formed under such conditions is not identical with glycogen (24); it is largely insoluble in cold water, gives a blue colour-reaction with iodine, and resembles in many ways the "synthetic starch" which has been obtained from the Cori ester by the action of potato-phosphorylase (25, 26, 27). When treated with β-amylase it is almost completely hydrolyzed to maltose. Like the amylose fraction of the natural starch, the synthetic polysaccharide is made up of long unbranched chains of *d*-glucopyranose units joined in 1 : 4 position.

In the liver, the Cori ester formed by phosphorolysis is acted upon by a phosphatase to yield free glucose. In presence of fluoride, this dephosphorylation is suppressed and the Cori ester is converted to 6-phosphohexose.

Phosphoglucomutase.—This is the enzyme which catalyzes reversibly the shift of the phosphate group of phosphoglucose from position C_1 to C_6 with the subsequent formation of the Robison ester or $d(+)$6-phosphoglucopyranose. It requires magnesium or manganese ions for its activity. If used in purified form, that is in absence of phosphorylase and isomerase, it sets up an equilibrium at which 4.8 per cent phosphoglucose is present as Cori ester at 20°, 5.5 per cent at 30°, and 5.8 per cent at 40°, the position of the equilibrium being independent of pH (28). The action of phosphoglucomutase on 6-phosphoglucose in conjunction with phosphorylase results in the formation of polysaccharide. If the enzymes are allowed to act on glycogen, 6-phosphoglucose is formed. A satisfactory agreement is found between the intensity of glycogen metabolism and the phosphorylase and phosphoglucomutase activity of various tissues. This finding favours the assumption that the metabolism of glycogen in all animal tissues follows the same path as in muscle (29).

Hexokinase (heterophosphatase).—Glucose as such cannot be built up into polysaccharide by phosphoglucomutase plus phosphorylase, unless it is first converted enzymically into 6-phosphoglucose. Adenosinetriphosphate (ATP) is the phosphate donator which yields the necessary phosphate group (30 to 34). To a small extent inosinetriphosphate can act as a substitute for ATP (35). The phosphate transfer from ATP to glucose is catalyzed by hexokinase, an enzyme which occurs abundantly in yeast. In presence of hexokinase ATP is converted to adenosinediphosphate (ADP) and a molecule of monophosphohexose is formed (36)

$$\text{ATP} + \text{hexose} \rightarrow \text{ADP} + \text{phosphohexose}$$

Myokinase.—Although ADP itself does not react with glucose in the presence of hexokinase alone, it can be made available for phosphorylation by the action of myokinase, a protein isolated from skeletal muscle (36). Myokinase is distinguished by its stability to high temperature at low pH and is unaffected by precipitation with trichloroacetic acid. It is inactivated by hydrogen peroxide but it regains its activity after reduction with cysteine or glutathione. According

to Kalckar (37) myokinase acts by catalysing the shift of a phosphate group from one molecule of ADP to another in the following manner

$$2\ \mathrm{ADP} \rightleftarrows \text{adenylic acid} + \mathrm{ATP}$$

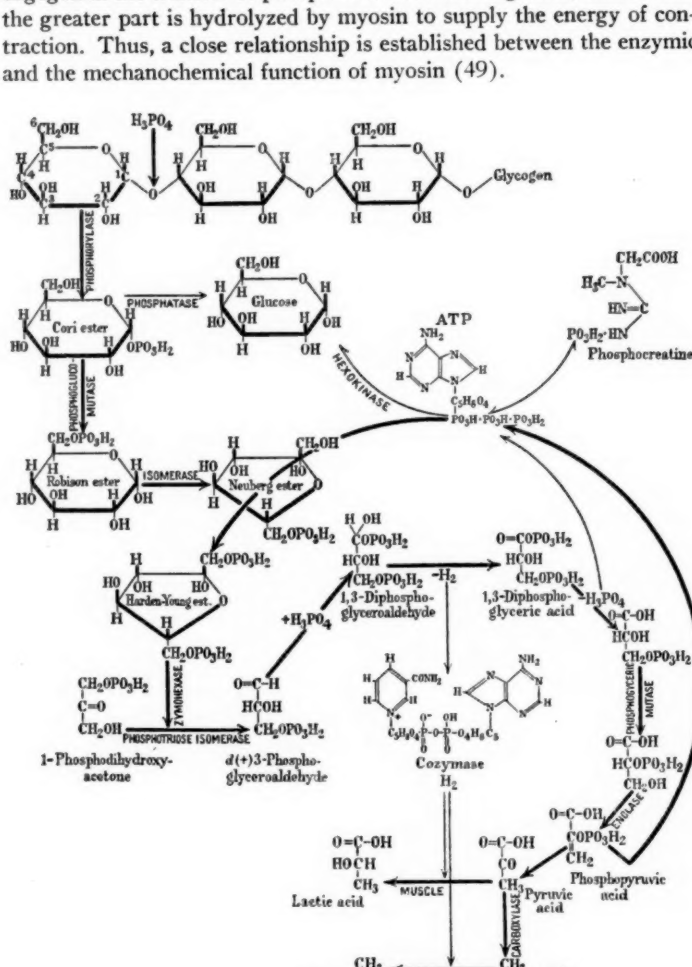
Thus if ADP is brought in contact with glucose in the presence of myokinase and hexokinase, it undergoes a "phosphate dismutation" and the ATP formed in this reaction can be utilized for the phosphorylation of glucose.

Phosphohexose isomerase (oxoisomerase).—This enzyme catalyzes the formation of an equilibrium mixture composed of Robison ester or $d(+)$6-phosphoglucopyranose (70 per cent) and Neuberg ester or $d(+)$6-phosphofructofuranose (30 per cent). The mixture of these two phosphohexoses is also known as "Embden ester" (muscle) or as the "Robison equilibrium ester" (yeast).

Enzymic formation of diphosphohexose. Role of ATP.—In the normal course of glycogenolysis the Neuberg ester is converted to the Harden & Young ester or $d(+)1:6$-diphosphofructofuranose, with ATP acting as the donator of phosphate (38, 39). The enzyme which brings about the transfer of phosphate is water-soluble but has not been isolated so far. The reaction between ATP and monophosphofructose is accompanied by considerable heat evolution and in this respect it recalls another reaction which also involves the breakdown of ATP, namely the hydrolysis of ATP to ADP and inorganic phosphate. This latter reaction is catalyzed by a specific phosphatase known as adenosinetriphosphatase.

The conception that the action of adenosinetriphosphatase on ATP provides directly the free energy of muscle contraction, received strong support when Engelhardt & Lyubimova discovered that pure myosin, the contractile element of the muscle fibres, can catalyze the dephosphorylation of ATP to ADP and inorganic phosphate (40, 41). This finding has since been confirmed and the mechanism of the reaction has been the subject of extensive studies (42 to 48). As pointed out by Needham (44) a substantial difference exists between the nature and function of the two enzymes involved in the dephosphorylation of ATP. Since the transfer of phosphate from ATP to the Neuberg ester is brought about by a soluble enzyme, it appears unlikely that the energy set free by the action of this enzyme could be utilized directly by the muscle fibres. Instead, this energy probably represents a part of the "waste heat" which is known to accompany the muscle contraction. The adenosinetriphosphatase on the other hand,

being located in the muscle fibres, can split ATP and provide energy directly to the contracting muscle. One may assume that during the anaerobic stimulation of muscle only a relatively small part of ATP is engaged in the transfer of phosphate to the Neuberg ester, while much the greater part is hydrolyzed by myosin to supply the energy of contraction. Thus, a close relationship is established between the enzymic and the mechanochemical function of myosin (49).



The enzymic activity of myosin is highest at pH 9. It is markedly enhanced by several cations, particularly by calcium (45). Magnesium and silver ions inhibit the enzyme (49). Magnesium acts by competing with calcium for adenosinetriphosphatase (50, 51). According to Du Bois *et al.* (50), this antagonism explains why the anaesthesia induced with magnesium sulphate is counteracted by calcium ions. However, Greville & Lehmann (51) disagree with this explanation.

Although myosin is invariably associated with the dephosphorylation of ATP, adenosinetriphosphatase activity is found in other tissues as well which, however, lack myosin. It is interesting to note that here, too, calcium can function as an activator of adenosinetriphosphatase. Du Bois & Potter (52, 53), who pointed out this fact, also made the observation that in the absence of calcium, acetylcholine stimulated the action of adenosinetriphosphatase.

Zymohexase (aldolase).—This enzyme splits the Harden & Young ester into a molecule of $d(+)$3-phosphoglyceraldehyde (Fischer-Baer ester) and a molecule of 1-phosphodihydroxyacetone. The enzyme has been isolated from muscle (54) as well as from yeast (55). Recently a crystalline enzyme preparation was obtained by Warburg & Christian (56) from rat muscles (1 gm. from 20 to 30 rats). They found that yeast, but not muscle, zymohexase is inactivated by metal-binding substances such as pyrophosphate, $\alpha\alpha'$-dipirydyl, cysteine, and glutathione, but can be restored to full activity by zinc, cobalt, copper, and ferrous salts.

The zymohexase content was determined in various rat tissues (57). Calculated per ml. of "undiluted cell substance" muscle contains 1460 μg., spleen 27 μg., erythrocytes 11.4 μg., and Jensen sarcoma 87 μg., zymohexase. Liver and kidney are poor sources of the enzyme. Blood plasma of normal rats was found to contain 0.28 μg. per ml. but in tumour-bearing rats considerably more was present, up to 2.28 μg. per ml.

Phosphotriose isomerase.—The action of zymohexase is followed by that of phosphotriose isomerase. Under the influence of this enzyme an equilibrium is reached at which much the greater part of phosphotriose is in the form of phosphodihydroxyacetone. The equilibrium values of both zymohexase and isomerase were determined by Meyerhof & Junowicz-Kocholaty (58) and were found to be unaffected by phosphate either directly or in the presence of the "oxidizing enzyme" of Warburg & Christian, with or without cozymase.

This the authors interpret in favour of the conception that the step which follows the action of isomerase, namely the phosphorylation of 3-phosphoglyceraldehyde to 1:3-diphosphoglyceraldehyde, should be regarded as a non-enzymic formation of a loose physical addition product and not as an enzymic reaction. However, the final answer must await the isolation or synthesis of 1:3-diphosphoglyceraldehyde. So far, only the dimeric form of this compound has been obtained synthetically (59) but the product was biologically inactive as it was not oxidised enzymically to the Negelein & Brömel ester or 1:3-diphosphoglyceric acid.

Phosphoglyceromutase.—The $d(-)$3-phosphoglyceric acid, formed by the dephosphorylation of 1:3-diphosphoglyceric acid, with adenylic acid as acceptor of the liberated phosphate group, is further converted by phosphoglyceromutase to $d(+)$2-phosphoglyceric acid.

Enolase.—This is the enzyme which catalyzes the reversible formation of phosphopyruvic from $d(+)$2-phosphoglyceric acid, both in muscle and in yeast. It has been purified from yeast by Warburg & Christian (60). A crystalline derivative of the enzyme protein was obtained in the form of a mercury complex. Magnesium forms an essential component of enolase. Among the intermediary fermentation reactions, the conversion of 2-phosphoglyceric acid into enolphosphopyruvic acid is the one most sensitive to fluoride, the inhibition being due to the formation of a magnesium-fluoro-phosphate complex. Enolase activity was also demonstrated by Utter & Werkman (61, 62) in a cell-free preparation from *Escherichia coli.* Like the action of the yeast enzyme, that of the bacterial enzyme was found to depend on magnesium or manganese ions.

Enzymes concerned with the breakdown of phosphopyruvic acid.—After phosphopyruvic acid, both in muscle and in yeast, the phosphate group leaves the chain of phosphorylated intermediates (63, 30, 64). It is taken up by acceptors such as ADP and adenylic acid, in yeast also by adenosine. The enzyme system engaged in the transfer of phosphate from phosphopyruvic acid requires, like enolase, the presence of magnesium or manganese ions, but in addition potassium also appears to play an essential role, with calcium acting as its antagonist (65, 66). The reconstituted ATP is subsequently made available for the phosphorylation of glucose, 6-phosphofructose, and creatine. Pyruvic acid liberated from phosphopyruvic acid is either reduced directly to lactic acid (muscle) or decarboxylated by carboxylase to acetaldehyde which in turn is reduced to alcohol (yeast).

The reduction of pyruvic acid and acetaldehyde is catalyzed by dehydrogenases which depend for their activity on dihydrocozymase formed from cozymase in the course of the oxidation of diphosphoglyceraldehyde to diphosphoglyceric acid.

Amylase.—It is now generally accepted that there are at least two possible mechanisms for the anaerobic breakdown of starch and glycogen, one associated with the action of phosphorylase, the other with an active participation of amylases. Both enzyme systems appear to be present in certain tissues, but it is not quite clear whether in a given tissue both can act in parallel or whether they exclude each other. The matter is further complicated by the circumstance that amylases, according to their origin, exhibit different properties. The blood diastase, for instance, is said to differ from other amylases in that it hydrolyzes glycogen and starch at nearly the same rate (67). Its level in blood is affected by the internal secretory glands (68, 69) and fluctuates in certain diseases (70, 71). Other amylases, including those in plants, are sensitive to a variety of factors such as certain inorganic salts (72), ascorbic acid (73), and a protein-like inhibitor present in grain (74).

The nature of the prosthetic group of amylase is unknown but the presence of free amino-groups in the enzyme protein has been found to be indispensable for the full activity of pancreas amylase (75).

Invertase.—New methods for the purification of invertase from yeast are described by Adams & Hudson (76). Their purest preparations contain a small amount of carbohydrate. Preparations from different yeasts and purified by different methods vary in their behaviour towards substrates (12). In addition to β-*d*-fructofuranosidase which hydrolyzes the glucose-fructose link in both sucrose and raffinose, they contain α-*d*-galactosidase, a small quantity of β-*d*-glucosidase, an inulase specifically concerned with the hydrolysis of inulin, and finally an enzyme defined as β-*d*-mannosidase which hydrolyzes β-phenyl-*d*-mannoside. The reactivating effect of sulphhydryl compounds on inactive invertase preparations was studied by Wagreich *et al.* (77). The effect of oxidants and reductants on disaccharases was investigated by Fiegenbaum (78).

Increasing attention has been paid lately to the enzymic synthesis of polysaccharides from sucrose. Cell-free enzyme preparations have been obtained from bacteria capable of synthesizing polyfructoside and polyglucoside from sucrose (79, 80).

Diffusing Factors

The early observations on the existence in the mammalian testicles of factors which profoundly modify the permeability of connective tissue (81, 82) and the later work which established the wide distribution in nature of similar factors as well as their enzymic character [bacteria (83, 84); malignant tissue (85, 86); snake venom (87); leeches (88)] opened up a new field of research into hitherto obscure biological processes. The pioneering work of a comparatively small group of scientists in England and in the U.S.A. enabled us to gain insight into the mechanism of the invasive power of certain virulent bacteria; it explained the reasons for the rapid spread in the body of poisonous snake venoms; it shed light on the phenomenon of reduction of viscosity in such biological fluids as the synovial fluid; and lately has made a new approach towards better understanding of the circumstances accompanying the fertilization of the mammalian egg.

The task of the reviewers of this subject is much lightened by the recent publication of an extensive review by Duran-Reynals (89) in which, although he emphasizes mainly the bacteriological aspects of the problem, the author quotes some 260 papers which cover practically the entire literature concerned. However, in order to obtain a more complete picture it is advisable to consult an earlier résumé by McClean (90) as well as a paper by Meyer (91) in which he discusses the chemistry of the substances involved in diffusing processes. The subject was last surveyed by Glick (91a).

It might be advantageous for those less familiar with the chronological developments of this research, to precede the discussion of the recent advances by a brief description of the terminology and assay technique in use, especially as the latter differs somewhat from the standard procedure in enzyme study.

To begin with there is the term "skin spreading factor" which originates from experiments designed to measure *in vivo* the increase in the permeability of the dermis. Several methods and modifications exist but the most accurate appears to be that of Bacharach, Chance & Middleton (92) which is based upon the measurement in rabbit skin of the total area through which a preparation of diffusing factor spreads in a given time, compared with the area of spread of a standard preparation. A more recent method (93) is based on the determination of the least amount of spreading factor required to produce a 20 per cent increase in the bleb area in guinea pig skin.

Next, there is "hyaluronidase," primarily used in connection with the enzymic hydrolysis of the mucopolysaccharide hyaluronic acid (94, 95) present in vitreous humour, synovial fluid, skin, umbilical cord, etc. The technique of hyaluronidase assay is twofold; it follows quantitatively the fall in viscosity due to the depolymerization of hyaluronic acid, and it also involves the determination of glucuronic acid and N-acetylglucosamine which are the breakdown products of hyaluronic acid. Much work has been done on the viscosimetric method which is beset with difficulties (96, 97, 98).

There is also the "mucin clot prevention (m.c.p.) test" in which a protein-hyaluronic acid complex is prevented by the action of the enzyme from forming a typical mucin clot on the addition of acetic acid. To emphasize the mucolytic nature of this process it has also been described as "mucinase" (99). A recent description of the m.c.p. test is given by McClean (100).

A remarkable feature is the suppression of the enzymic activity as manifested in the above tests, by specific antisera. Whether the enzymes be tested by the skin spread, the fall in viscosity, the clot prevention, or by the estimation of reducing sugars, all these activities can be neutralized by the addition of appropriate sera. The intriguing part is the high specificity shown in these reactions. Thus, for example, sera against bacterial hyaluronidase are species-, but not type-, specific, while serum against diffusing factor from bull's testis inhibits this enzyme but is powerless against one made from mouse testis or a bacterial enzyme (100).

Chain & Duthie (101, 102) were the first to show that fairly pure preparations of testicular spreading factor also possess hyaluronidase activity. Since then much work was done to establish more precisely the relationship between the skin spreading effect and the mucolysis of hyaluronic acid. The problem was attacked in various ways but mainly by examining and comparing the properties of enzymes obtained from widely differing sources as well as by testing the activity of a given preparation by means of all the above-mentioned assay methods. An up-to-date discussion of this topic will be found in a paper by McClean (100) and by Lythgoe & Madinaveitia (103). From the evidence so far available one is disposed to believe that the factor responsible for the diffusion in the dermis and the hyaluronidase activity, as displayed in the viscosimetric and clot prevention tests, are identical; the final answer, however, must await the complete purification of enzymes from several separate sources. A claim has re-

cently been made (104) that a purified mucinase preparation from testis is nitrogen-free and yet possesses about half the activity of the original extract, a result which obviously calls for substantiation from other investigators in this field.

The following scheme illustrates the successive stages in the degradation of hyaluronic acid and its breakdown products.

Substrate	Active Agent		Characteristics
Viscous hyaluronic acid	Testis (81, 82) Bacteria (83, 84) Snake venoms (87)	Enzymic	Rapid, optimum pH 6.0 to 7.0 dependent on salts. Anaerobic, insensitive to organic solvents, antiseptics.
↓	Diazo-compounds (113, 115) Ascorbic acid (114) Copper salts (116)	Non-enzymic	Independent of pH
Depolymerized hyaluronic acid	Testis (81, 82) Bacteria (83, 84) Venoms (87)		Slow
↓			
Glucuronic acid + N-acetylglucosamine ↓ Glucosamine + acetic acid	Testis (105) Kidney (105) Brain (105) Bee venom (105) Bacteria, Yeast (105)		Fairly rapid, optimum pH 7.4 to 7.8, sensitive to organic solvents, antiseptics. Aerobic in animal tissues and bee venom; anaerobic and aerobic in yeast and bacteria. Glucosamine but not N-acetylglucosamine deaminated by ascorbic acid + copper.*
↓ Ammonia + deamination product			

* C.L.-M., unpublished work.

Despite the uncertainty as to the identity or otherwise of skin spreading factors and hyaluronidases, several important contributions were made particularly regarding the question of tissue permeabilization by pathogenic microorganisms. Of considerable interest is the "adaptive" capacity of certain bacteria which react to the inclusion of hyaluronic acid in the medium with the production of increased amounts of hyaluronidase. On the other hand, capsulated strains cultivated in a hyaluronidase-containing medium fail to develop capsules while in this medium but do so again as soon as they are

transferred to an ordinary medium (106, 107). When, however, attempts were made to decapsulate streptococci *in vivo,* striking differences came to light between the *in vitro* and *in vivo* activity of hyaluronidase, as it was found that there is an inhibitor of the enzyme in the blood of the living animal, apparently linked with the pseudoglobulin fraction of the serum (108). Other hyaluronidase inhibitors are chondroitin sulphuric acid, heparin, gastric mucin (none of which are attacked by the enzyme), and depolymerized hyaluronic acid, but not glucuronate or glucosamine. The inhibition due to these substances is probably of the competitive type as the inhibitors are structurally similar to the substrate. It is worth mentioning that drugs of the sulphonamide group have no effect upon hyaluronidase (100).

The part played by sperm hyaluronidase in fertilization was investigated by McClean & Rowlands (109). The cumulus cells and the corona radiata of the rat egg are embedded in a transparent viscous gel which must be removed before the sperm can enter the egg. Hyaluronidase prepared from various sources (testis, bacteria, snake venom) was allowed to act on the gel and its effect on it was followed microscopically. The authors recorded the time taken by the enzymes investigated to disperse the cumulus cells and the corona radiata, the end-point of the reaction being the complete denudation of the egg. The enzyme action was found to be restricted to the liquefaction of the gel (which probably contains hyaluronic acid) without affecting the ovum itself. Pretreatment of the cell masses with sodium glycocholate prevented hyaluronidase from attacking the gel, even after thorough washing away of the detergent. The authors in discussing the implications of their observations, indicate the possible reasons for certain types of sterility as being due either to an insufficient concentration of hyaluronidase as in instances of low sperm count, or else to an actual deficiency in the formation of the enzyme by the male organism. The dispersing effect of hyaluronidase on the follicle cells surrounding the mouse ovum was confirmed by Fekete & Duran-Reynals (110).

In two papers published in the medical press McClean and his collaborators (111, 112) lead the way towards a practical application of the knowledge acquired in hyaluronidase studies. They give details for a method by means of which, with the help of suitable antisera, it may be possible to diagnose the presence of gas-gangrene organisms in wounds at a stage when infection cannot be recognized by the routine bacteriological examinations. The technical side is

worked out in such a manner as to make the method accessible to the medical attendant under service conditions.

The story would not be complete without brief reference being made to the existence of diffusing factors of non-enzymic character. Favilli (113) gives a description of the mucolytic properties of an azoprotein prepared from diazobenzenesulphonic acid coupled with horse serum. Such a substance is capable of reducing the viscosity of synovial fluid but the rate of activity differs from that shown by hyaluronidase; unlike the enzyme, the artificial factor is hardly influenced by pH. In this group falls the discovery of Robertson, Ropes & Bauer (114) that ascorbic acid in the presence of hydrogen peroxide brings about a degradation of synovial mucin which, however, is not accompanied by the liberation of reducing sugar. Unlike the natural mucinase, ascorbic acid acts on the gastric and salivary mucins as well as on starch, pectin, and cartilage. Several experiments on the effect of ascorbic acid and of diazo-compounds are recorded in an earlier paper by Madinaveitia & Quibell (115). An interesting observation was made by Pirie (116) who isolated a viscous polysaccharide from a fowl tumour devoid of hyaluronidase activity. She found that the preparations of this mucopolysaccharide contained varying amounts of copper. Moreover, the addition to hyaluronic acid of traces of copper catalyzed the reduction of viscosity, the copper effect being blocked by the addition of diethyldithiocarbamate.

A study of the antigenic properties of hyaluronic acid was carried out recently by Humphrey (117) who coupled the mucopolysaccharide with a horse serum protein by azobenzyl ether linkages. However, even in this combination, hyaluronic acid failed to provoke antibody formation in the rabbit.

Enzymes Concerned with the Utilization, Liberation, and Transport of Carbon Dioxide

Since 1935 when the concept of carbon dioxide utilization in the metabolism of heterotrophic organisms was first established by Wood & Werkman, considerable advances have been made towards a better comprehension of the mechanism of carbon dioxide fixation by cells. Evidence now available shows that carbon dioxide enters the metabolic processes in the heterotrophic organisms in conformity with the equation

$$CO_2 + \text{pyruvic acid} \rightleftharpoons \text{oxaloacetic acid}$$

However, attempts to isolate the enzymes responsible for carbon dioxide assimilation are still in an early stage. Using cell-free enzyme preparations from pigeon liver Evans *et al.* (118, 119) obtained fixation of $C^{11}O_2$ which enters into the reaction with pyruvic acid to form oxaloacetic acid. Several studies were made with enzymes extracted from microorganisms (120, 121, 122).

The extract from *E. coli* attacks pyruvic acid anaerobically and produces from it carbon dioxide, acetic, formic, lactic, and succinic acid. It is active within a pH range of 6.2 to 7 and can be dried without loss of activity. Inorganic phosphate, manganese, and cocarboxylase are essential components of this system. According to Utter & Werkman (123) inorganic phosphate is utilized for the formation of an intermediary compound resembling acetylphosphate; when adenylic acid is available as phosphate acceptor, adenosinetriphosphate accumulates. Kalnitsky *et al.* (121, 122) while studying the anaerobic dissimilation of pyruvate by the enzyme system extracted from *E. coli,* found that occasionally the carbon dioxide evolution fell far short of the values usually obtained, although identical quantities of pyruvate had disappeared. This proved to be due to the fixation of carbon dioxide. In the presence of $C^{13}O_2$ a significant amount of heavy carbon appeared in the carboxyl group of succinic acid. Since the primary product of fixation must have been oxaloacetic acid with C^{13} in the carboxyl group adjacent to the methylene group (120), the formation of succinic acid can be formulated as follows:

$$C^{13}O_2 + CH_3 \cdot CO \cdot COOH \rightarrow HOOC^{13} \cdot CH_2 \cdot CO \cdot COOH$$

$$HOOC^{13} \cdot CH_2 \cdot CO \cdot COOH + 4\,H \rightarrow HOOC^{13} \cdot CH_2 \cdot CH_2 \cdot COOH + H_2O$$

Yeast carboxylase.—The purest of all decarboxylating enzymes so far obtained is the carboxylase from yeast on which further studies are reported (124, 125, 126). The activity of this enzyme requires the presence of cocarboxylase (diphosphothiamin) of the formation of which little as yet is known. It is, however, interesting to note that yeast contains enzymes which hydrolyze and synthesize cocarboxylase (127). The existence of a factor in the flesh of certain kinds of fish which destroys thiamin was brought to light when it was found that foxes fed on raw fish develop polyneuritis (Chastek paralysis). The factor responsible for the destruction of the vitamin was found to be an enzyme which is present in nearly every

organ of certain fishes, particularly in the liver, spleen, intestine, and gills (128). It is common in fresh-water fish but absent in the tissues of several salt-water fish (129).

Acetoacetic acid decarboxylase.—The purification of a new enzyme, the acetoacetic decarboxylase, from *Clostridium acetobutylicum* was accomplished by Davies (130). The enzyme acts specifically on acetoacetic acid and converts it to acetone. It is optimally active at pH 5 and requires the presence of a coenzyme, probably riboflavin-phosphate.

Carboxylases of animal tissues.—These enzymes have been extensively investigated by Green *et al.* (131). They obtained from minced pig heart an insoluble protein fraction which in the presence of diphosphothiamin and magnesium or manganese ions constitutes an enzyme system capable of anaerobic decarboxylation of α-ketoacids. Under the action of this enzyme pyruvic acid is decarboxylated to acetoin, α-ketobutyric acid to propioin, and α-ketoglutaric acid to succinic semialdehyde.

Amino acid decarboxylases.—Enzymes concerned with the decarboxylation and deamination of serine, threonine, cysteine, cystine, and arginine by *Clostridium welchii* have been studied by Woods & Trim (132). Bacterial amino acid decarboxylases were investigated by Gale & Epps (133 to 136) in conjunction with a general study on the enzyme formation and enzymic processes in microorganisms. Under suitable growth conditions specific enzymes are formed in bacteria which bring about the decarboxylation of *l*(+) arginine, *l*(+) ornithine, *l*(—) histidine, and *l*(+) lysine with the formation of agmatine, putrescine, histamine, and cadaverine, respectively. A purified preparation of *l*(+) lysine decarboxylase was made from acetone-dried powders of a paracolon organism, *Bacterium cadaveris* (136). The enzyme is optically specific and can be used for the resolution of *dl*-lysine and the preparation of *d*-lysine as well as for a rapid and specific estimation of *l*-lysine. The enzyme is most active at pH 6. It requires a coenzyme hitherto unidentified but widely distributed in nature. A concentrated solution of this coenzyme is deep yellow but it shows no flavine-like fluorescence; it gives a well defined lead salt containing 5.07 per cent nitrogen and 3.02 per cent phosphorus.

The presence of an *l*(—) cysteic acid decarboxylase in liver extracts of dog, rat, pig, and guinea pig has been demonstrated by Blaschko (137). The enzyme was also found in kidneys (138). It produces taurine from *l*(—) cysteic acid, is reversibly inhibited by

cyanide, and shows many similarities to $l(-)$ DOPA decarboxylase, an enzyme which occurs in the liver and kidneys of many mammals and which forms hydroxytyramine from $l(-)$ 3:4-dihydroxyphenylalanine (139, 140).

Carbonic anhydrase.—Most of the carbon dioxide given up by the tissues of man and higher animals is carried to the lungs by the blood plasma in the form of bicarbonate. Both the rate with which carbon dioxide is taken up by the blood, as well as the rate with which it is expelled from the lungs, depend on the velocity of the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

Spontaneously this reaction would proceed so slowly that only a small proportion of the carbon dioxide known to be given off in the lungs would be liberated in the brief period during which the blood passes through the lung capillaries. The proper rate of reaction, however, is assured by the presence in the red cells of carbonic anhydrase. It was shown by Keilin & Mann (4, 141) that pure carbonic anhydrase is a zinc-protein compound where zinc forms the active part of the enzyme molecule. This was since confirmed by Hove *et al.* (142) and Scott *et al.* (143, 144, 145). The last mentioned workers claim that their purified preparations are more active than those of Keilin & Mann who, however, pointed out recently (146) that this discrepancy is only apparent and is due to a different way of assessing the activity of carbonic anhydrase and furthermore, to the use by Scott and his collaborators of peptone as a "stabilizer" which, although it doubles the activity of purified preparations, has no effect whatever on the carbonic anhydrase activity of plasmolysed blood. The homogeneity of the pure enzyme of Keilin & Mann has been confirmed by experiments with electrophoresis (4) and ultracentrifuge (147). Similar studies carried out by Petermann & Hakala (148) with the preparation of Scott & Fisher revealed a small impurity. The relation between zinc and the activity of carbonic anhydrase has been investigated by Main & Locke (149).

The mode of action of carbonic anhydrase within the intact blood cells has been elucidated by Keilin & Mann (150) who made use of the following facts: (*a*) hemoglobin can be oxidised to methemoglobin within the red cells; (*b*) methemoglobin can serve as an indicator changing its colour and absorption spectrum from that of acid methemoglobin at pH 6.5 to that of alkaline methemoglobin at pH 9; and (*c*) the activity of carbonic anhydrase as found by Mann &

Keilin (151), can be specifically abolished by sulphanilamide. By making use of these facts and by employing a spectroscopical method, Keilin & Mann determined the velocity of the pH changes within the "methemoglobin corpuscles" and found that it is considerably reduced in the presence of sulphanilamide. With the help of the same technique they also demonstrated that sulphanilamide specifically inhibits the shift of the chloride ion to and from the red blood cells. In this way it was possible to prove that the activity of carbonic anhydrase, which is located within the blood cells, is intimately linked through ionic exchange reactions with the acid-base changes which take place in the plasma. The existence of such a link was confirmed by Fegler (152) who, in addition, demonstrated that carbonic anhydrase can catalyze both the evolution and the uptake of free carbon dioxide in intact blood. The role of carbonic anhydrase in ionic exchanges involving the erythrocytes was studied by Jacobs & Stewart (153). A paper by Free *et al.* (154) deals with the relation which exists between the inhibition of carbonic anhydrase by sulphanilamide and the occurrence of acidosis in blood as well as the bicarbonate excretion in urine following the administration of sulphanilamide. Stevenson (155) reports that the concentration of carbonic anhydrase in the blood of new-born infants is less than half, and in prematurely born infants one-fourth, of that found in adults, and that the enzyme level is markedly lowered in cases of cyanosis.

It is significant that carbonic anhydrase is closely associated with organs which are in control of the acid-base equilibrium of the body. It is located in the blood corpuscles, in the gastric mucosa, in the kidneys and in the pancreas. In Crustaceans the enzyme was found in the gills (156).

Proteolytic Enzymes

Herriot in the preceding volume of the *Annual Review of Biochemistry* gave a detailed account of the recent progress in this field. Since then, a new protease, hurain, has been isolated (157) from the sap of *Hura crepitans* (Euphorbiaceae). Rennin (rennet, chymosin) has been prepared in crystalline form by Berridge (158). Crystalline rennin is capable of clotting in 10 minutes at 37° approximately 10^7 times its dry ash-free weight of reconstituted milk, in the presence of calcium. Several of the recent papers deal with the distribution and function of proteolytic enzymes, notably in the thyroid gland

(159), brain (160), prostata (161), pancreas (162), and also in the salamander gastrula (163). Publications concerned with the mechanism of activation and inhibition of proteolytic enzymes include a study of the inactivating effect of ultraviolet radiation on trypsin (164), of the activating influence of hydrogen cyanide on papain (165), a description of "pancreozymin," a thermostable stimulant extracted from the small intestine which enhances the secretion of pancreatic enzymes (166), and a study of the effect of caffeine on digestive enzymes (167). In addition, there is some work on the action of proteolytic enzymes on fibrinogen (168, 169, 170), on serum pseudoglobulin and diphtheria antitoxin (171), on glutathione (172, 173), and on the release of histamine from blood cells (174). Proteolytic agents were found to participate in the conversion of the atoxic ε-prototoxin into the active ε-toxin of *Clostridium welchii* type D (175, 176). Plasteins, insoluble in dilute trichloroacetic acid, have been obtained by the action of trypsin and papain on digestion products of insulin (177).

Renin.—Attention was focused lately on renin, an enzyme which has its origin in the kidneys and which acts on a globulin present in the circulating blood, with the production of a heat-stable, dialysable, vasoconstrictor and pressor substance known as angiotonin or hypertensin (179 to 183). Several methods of preparation and purification of renin from hog kidneys have been described; the more recent ones are those of Schales (184) and Katz & Goldblatt (185); the purest renin preparation so far reported has an activity of 130 dog units per mg. enzyme-nitrogen, one dog unit representing the amount which raises the blood pressure by at least 30 and not more than 35 mm. Hg within three minutes in at least three unanaesthetized dogs (186).

The serum globulin which forms the substrate for renin has been variously described as preangiotonin, prehypertensin, hypertensin precursor, hypertensinogen, and renin activator (187, 188). Its nature has been established as that of an α_2-globulin component of the pseudoglobulin fraction of serum (189, 180). Both renin and its substrate are inactive by themselves, whereas angiotonin is highly active but can be inactivated by angiotonase (hypertensinase), an enzyme which occurs in several organs and in the blood (190, 191). A method has been devised for the quantitative assay of renin in presence of angiotonase (192). The enzymic formation and destruction of angiotonin are presumably balanced in normal animals. When however, this balance is disturbed, hypo- or hypertension may result.

Amidases

Urease.—The recent contributions on urease are mostly concerned with the action of various inhibitors of this enzyme. Elson (193) states that very dilute solutions of ascorbic acid completely prevent the formation of ammonia while even a one in a million solution causes 50 per cent inhibition. This inhibitory effect is abolished by the addition of cysteine. Quastel (194) points out that the ascorbic acid effect is probably due to traces of dehydroascorbic acid. He quotes his older work on the inhibitory effect on urease of very dilute catechol or quinol which he found was due to small amounts of quinone in the phenol solutions which, after reduction with cysteine, lost their inhibitory power. Highly purified specimens of penicillin also suppress urease activity (195). The observation is of interest as instances were recorded in the medical literature of elevated blood urea in some penicillin-treated patients. Hellerman, Chinard & Deitz (196) made a thorough study of the reversible inactivation of urease by reagents known to act on the sulphhydryl groups, such as organic mercaptide-forming salts like *p*-mercuribenzoate, oxidizing agents like porphyrindin, iodosobenzoate, and an alkylating agent iodoacetamide. They claim that there are differences in the chemical reactivity among the sulphhydryl groups of urease which may cause functional differences. Also, some groupings are more, others less, readily accessible to the action of the various substances investigated. Mueller & Rusch (197) studied the effect on urease of benzopyrene in caffeine solution which had been exposed to ultraviolet radiation for one hour. The enzyme was almost completely inactivated. Fresh benzopyrene had no effect and irradiated caffeine alone inhibited but slightly. The effect is explained as being due largely to hydrogen peroxide formation and in addition, to a product formed from benzopyrene during the irradiation with ultraviolet light.

Arginase.—A study of arginase activity in the liver after adrenalectomy was carried out (198) and revealed a definite decrease in the activity of the enzyme which, however, could be stimulated by certain adrenal steroids, like corticosterone, 11-dehydrocorticosterone, and 11-dehydro-17-hydroxycorticosterone. On the other hand, desoxycorticosterone and male and female sex hormones were without influence on arginase activity. Rossi (199) subjected crude arginase from hog liver to prolonged dialysis and found that the enzyme became inactive but could be reactivated by the addition of the dialysate.

The author is of the opinion that manganese ions only enhance the arginase activity but he rejects the idea of manganese being an actual constituent of the enzyme.

Histidase and urocanase.—Edlbacher & Viollier (200) describe the preparation and separation from each other, of liver histidase and urocanase. Purified histidase is inhibited by urocanic acid but urocanase is not affected by *d*- or *l*-histidine or by pyruvic acid. Both enzymes are present in human liver. A study of these enzymes during pregnancy failed to show an increase in the liver histidase activity. This fact, in the authors' opinion, contradicts the theory that pregnancy histidinuria is due to an interference with histidase activity by prolans.

Cysteine Desulphurase

The enzyme which splits cysteine into hydrogen sulphide, ammonia, and pyruvic acid appears to be widely distributed. New studies on cysteine desulphurase are mainly concerned with the mechanism of the reaction. Smythe & Halliday (201) using rat liver sought to establish conditions in which to demonstrate the reversibility of the reaction. To the enzyme preparation they added hydrogen sulphide containing radioactive sulphur together with a certain amount of cysteine. When the nitrogen of part of the added cysteine had been converted into ammonia, the reaction was stopped, the remaining cysteine isolated and shown to contain appreciable amounts of radioactive sulphur. However, in experiments where hydrogen sulphide alone was used, no such results could be obtained. While, therefore, these experiments indicate a possible mode of cysteine resynthesis, the mechanism of the reaction remains obscure and awaits elucidation by further investigation.

Binkley (202) studied cysteine desulfurase in yeast and bacteria from which he obtained crude preparations which were really a mixture of yet another two enzymes, the serine deaminase and the enolase. He found that glucose, serine, and phosphoglyceric acid prevented the enzyme from attacking cysteine, while cysteine or serine inhibited the conversion of phosphoglyceric to phosphopyruvic acid. All these reactions led to pyruvic acid as end-product and all were sensitive to fluoride. On dialysis the enzyme preparation lost much of its activity which could be restored with traces of zinc, manganese, and magnesium salts.

Phosphatases

Several contributions dealing with various aspects were made recently in this field. Sizer's work (203) adds to our information about the effect which oxidants and reductants exert on enzymes. He finds that the activity of both the alkaline and the acid phosphatase (beef lung, kidney, milk, intestine) is but little affected by reducing agents. Mild oxidants interfere only to a slight degree but with the rise of the oxido-reduction potential beyond + 400 millivolt oxidants exhibit a strong inhibitory effect. This however, is mostly reversible either by dialysis or by the addition of a suitable reductant. An accompanying study of the ultraviolet absorption spectrum suggests to the author that it may be the tyrosine, but not the tryptophane, portion of the enzyme molecule which is affected by the oxidizing agents. Dounce (204) reports the presence of both alkaline and acid phosphatase in the isolated cell nuclei of rat liver, together with certain other hydrolytic and oxidative enzymes.

Schmidt & Thannhauser (205) set out to purify the alkaline phosphatase of intestine with a view of utilizing it as a tool for the structural elucidation of organic phosphorus compounds. Their final enzyme preparation gives a biuret and Molisch test. After acid hydrolysis it reduces alkaline copper revealing the presence of relatively large amounts of polysaccharide. The authors give as yet no indication as to whether, and in what way, the polysaccharide is essential for the enzyme activity. Small amounts of cysteine and cyanide inhibit, fluoride has no effect, magnesium salts activate slightly, while zinc, bile salts, and amino acids have no influence upon the enzyme. The purified intestinal phosphatase was active towards adenosinetriphosphoric acid, phosphopyruvic acid, pyrophosphoric acid, diphenylphosphate, and yeast nucleic acid, but it did not attack phosphatides, thymonucleic acid, or casein. Lawrie (206) analyzed the stools of twenty subjects and found considerable variability in their phosphatase content. The faeces phosphatase, which has its origin in the mucous membrane of the intestine, was but slightly affected by magnesium, inhibited by cyanide, and not influenced by fluoride. The effect of nutritional magnesium-deficiency in rats on the serum phosphatase was investigated by Snyder & Tweedie (207) who state that a fall in the serum-magnesium content to the level of 0.8 mg. per cent is followed by a noticeable and permanent decrease in serum phosphatase activity. A rise in serum phosphatase was shown in

hyperthyroid dogs to occur at a time when impairment of liver function due to the feeding of thyroid, could be demonstrated by the bromosulphalein retention test (208). A sharp increase in the activity of phosphatase in plasma was also recorded in rats with obstructive jaundice (209).

The dephosphorylation of cocarboxylase and of α- and β-glycerophosphate in yeast was found to be inhibited by aneurin and related substances but animal phosphatases were not similarly affected (210). The β-glycerophosphate activity of rat liver cancer produced by feeding of *p*-dimethylaminoazobenzene was about ten times that of normal rat liver. *In vitro,* however, neither the cancerous nor the normal liver phosphatase were affected by *p*-dimethylaminoazobenzene (211). The effect of salicylate on bone phosphatase both *in vitro* and after salicylate administration was investigated by Lutwak-Mann (126) using a large variety of phosphoric acid esters. However, the activity of the phosphatases in the rat bone extract was practically unaltered by salicylate.

The question of the existence of a specific hexosediphosphatase, distinct from other alkaline phosphatases, was investigated by Gomori (212), who obtained from liver and kidney an enzyme which at an alkaline pH and in the presence of magnesium dephosphorylated hexosediphosphate but was inactive towards other phosphoric esters. Without magnesium the hexosediphosphatase was inactive; cyanide stimulated and fluoride inhibited its activity. Adenosinetriphosphatase has been discussed on page 29.

Ohlmeyer (213) examined the phosphatase of the prostatic gland and of urine and found that both contain magnesium. The enzyme was sensitive to fluoride, which sensitivity is due to the formation of a dissociable complex with both magnesium and the enzymic protein.

The histochemical technique for the demonstration of phosphatases in tissues has been further improved (214). The study of alkaline and acid phosphatase in the nervous system (215, 216) showed the presence of marked phosphatase activity in the vascular endothelium of the central nervous system. It was also present in nervous tumour tissue and in areas of astrocytosis such as the chronic plaques in multiple sclerosis. Histochemical studies on the distribution of alkaline phosphatase were carried out by Bourne (217). In the alimentary tract of the guinea pig the enzyme was located where active glucose absorption is known to take place. He found also that ascorbic acid deficiency did not affect the phosphatase content of soft tissues but

that there was a decrease in the bones. Slight injury had no effect on the distribution of the enzyme. Use was made of the histochemical method in investigations on the alkaline phosphatase in the hydronephrotic kidney (218) and in the spinal cord of chick embryo (219).

Choline Esterase

The abundance of choline esterase in animal tissues and the ease with which its activity can be assayed, added to the general physiological interest of the problem, always assure a steady flow of research dealing with various aspects of this enzyme. Most of the recent investigations are concerned with the substrate specificity and the distribution of choline esterase in nature.

Richter & Godby Croft (220) show the existence of considerable differences in the properties of choline esterase present in the tissues of different species. Also within the same species the enzyme in the red cells is by no means identical with that present in serum. Thus, the human serum esterase acts not only on choline esters but on methyl butyrate as well, while the enzyme of the red cells hydrolyzes only choline esters. Quinine suppresses the enzyme action on acetylcholine but it does not inhibit the hydrolysis of methyl butyrate.

The team of Mendel, Mundell & Rudney (221 to 224) hold that the enzyme commonly called "choline esterase" in blood, serum, and certain tissues, is not specific in that it acts not only on choline but on other esters as well. The brain enzyme, however, is specific for choline esters. This enzyme they call the "true choline esterase," while for the others they suggest the name "pseudo-choline esterase." A method for the purification of the pseudo-choline esterase from pancreas is described (222). The following differences exist between the true and the pseudo enzymes. The true choline esterase requires a low substrate concentration and is inhibited by excess of substrate; the pseudo-choline esterase acts best at high substrate concentrations. Furthermore, the true, but not the pseudo-, choline esterase acts on acetyl-β-methylcholine, whereas benzoylcholine is attacked only by the pseudo-choline esterase. The examination of choline esterase in the brains of the representatives of all the vertebrates revealed the presence in this tissue of the true esterase only. On the other hand, the various other organs were found to contain varying amounts of both enzymes; moreover, the same organ of one species frequently differed in its type of choline esterase from that of another species.

Eadie (225) presents a study of the inhibition of choline esterase

by physostigmine and prostigmine. The inhibition is competitive in character and an examination of the equilibrium established between the enzyme and the inhibitor indicates that two molecules of the inhibitor are required for each active group of the enzyme. The kinetics of the system choline esterase-physostigmine were also dealt with by Strauss & Goldstein (226).

Several papers describe the effect of various substances upon the activity of choline esterase. Salicylate even in comparatively large amounts scarcely affected the serum choline esterase (126). Torda (227) studied several hormones among which progestin, estrone, follutein, testosterone, and pitressin, but not pitocin, all had a slightly lowering effect on choline esterase activity. Cystine, ascorbic acid, and fumaric acid inhibit choline esterase; also dyes such as methylene blue, safranine, Nile blue, and Janus green act as inhibitors. Procaine and tutocaine suppress the activity of the enzyme but this effect is not relieved by *p*-aminobenzoic acid (228, 229). Bloch (230) examined choline esterase and serum lipase in the presence of cresyl phosphates. He found that only the *o*-compound acted as inhibitor, the *m*- and *p*- derivatives being devoid of influence. Tri-*o*-chlorophenyl phosphate had an effect similar to that of tri-*o*-cresyl phosphate with which it is isosteric.

A survey of the distribution of choline esterase in the primitive nervous system by Bullock & Nachmansohn (231) revealed the existence of certain low organisms entirely devoid of choline esterase. However, significant amounts were found in *Hydrozoa,* coexisting with the appearance of a more highly differentiated nervous system. In *Platyhelminthes,* which possess nervous ganglia, the enzyme is present in a concentration far above that found in *Ecchinodermata.* Means (232) describes the presence of choline esterase in the nervous system of the grasshopper *Melanoplus differentialis.* The studies on the amphibian eye (233) indicate the presence of choline esterase in relatively high concentrations in the cornea, whereas none is found at the nerve endings in the pupillary sphincter. In the turtle (234) there is a powerful choline esterase in the nerve terminals of the pupillary sphincter which responds only to high concentrations of acetylcholine. The content of choline esterase was investigated in relation to the various stages of development in *Amblystoma punctatum* (235, 236) by means of a sensitive microtitration method. The results showed a close correlation between the enzyme activity and the functional ability as expressed by behaviour manifestations. The

functional capacity of larvae reared in eserine, prostigmine, or acetylcholine was profoundly affected. On removal from the inhibitor, recovery of the physiological and the enzymic activity were found to go parallel. It is concluded that the content in *Amblystoma* of choline esterase is a biochemical criterion of the functional capacity of the neuromuscular apparatus.

The formation of acetylcholine in the peripheral nervous tissue has been demonstrated by Feldberg (237) who found that acetylcholine released from sympathetic ganglia and cholinergic nerves by chopping is restored in the incubated chopped tissue up to the original level. The reaction no longer takes place if the nerves have degenerated following section forty-eight hours previously. Sensory roots were devoid of synthetic power.

An enzyme in human serum capable of splitting procaine to produce *p*-aminobenzoic acid was named procaine esterase (238, 239). It is sensitive to fluoride but not to cyanide. Apart from serum, the enzyme was also located in the liver and in the white of egg, but not in the chicken serum; in toxic goitre extraordinarily high values were observed, but following the removal of goitre there was a return to normal. On the other hand, low values prevailed in cases of malignant diseases of the digestive tract.

Lysis of Red Cells

An interesting observation on the mechanism of lysis of the red cells was made by Maegraith & Findlay (240). Tissues such as lungs, liver, kidney, spleen, marrow, and muscle will lyse saline suspensions of red cells. The lytic effect only takes place if the tissues themselves had been thoroughly washed in saline prior to incubation. Tissue slices incubated with unwashed red cells fail to lyse. The lysis can be suppressed by the addition of serum, by cyanide even in a 1 to 1,000,000 dilution, and by mercuric chloride 1 to 32,000. Heating the tissues to 80° destroys their lytic power. The system responsible for lysis, but not the serum inhibitor, was found to be species specific. The authors assume that they are dealing with an enzyme present in various tissues capable of destroying the red cells but normally held in check by the serum inhibitor. But blackwater fever and certain lytic anaemias may be instances where the balance between the two is disturbed.

LITERATURE CITED

1. Northrop, J. N., *Crystalline Enzymes*, pp. 31, 65 (Columbia Univ. Press, New York, 1939)
2. Borgstrom, E., and Koch, F. C., *Proc. Soc. Exptl. Biol. Med.*, **52**, 131–32 (1943)
3. Keilin, D., and Mann, T., *Nature*, **143**, 23–24 (1939)
4. Keilin, D., and Mann, T., *Biochem. J.*, **34**, 1163–76 (1940)
5. Guzman Barron, E. S., and Singer, T. P., *Science*, **97**, 356–58 (1943)
6. Hellerman, L. C., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, **147**, 443–62 (1943)
7. Johnson, F. H., Eyring, H., and Williams, R. W., *J. Cellular Comp. Physiol.*, **20**, 247–68 (1942)
8. Friedman, M., and Kaplan, A., *J. Exptl. Med.* **77**, 65–69 (1943)
9. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **145**, 61–68 (1942)
10. Bolomey, R. A., and Allen, F. W., *J. Biol. Chem.*, **144**, 113–19 (1942)
11. Loring, H. S., and Carpenter, F. H., *J. Biol. Chem.*, **150**, 381–88 (1943)
12. Adams, M., Richtmyer, N. K., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1369–80 (1943)
13. Buskirk, H. H., and Delor, R. A., *J. Biol. Chem.*, **145**, 707–8 (1942)
14. Allinson, M. J. C., *J. Biol. Chem.*, **147**, 785–91 (1943)
15. Bunting, A. H., *Biochem. J.*, **36**, 639–40 (1942)
16. Evans, J. S., and Hauschildt, J. D., *J. Biol. Chem.*, **145**, 335–39 (1942)
17. Cohen, S. S., and Stanley, W. M., *J. Biol. Chem.*, **142**, 863–70 (1942)
18. Kosterlitz, H. W., *Biochem J.*, **37**, 322–26 (1943)
19. Kagan, B. O., Lyatker, S. N., and Tsvasman, E. M., *Biochimia*, **7**, 93–108 (1942)
20. Doudoroff, M., Kaplan, N., and Hassid, W. Z., *J. Biol. Chem.*, **148**, 67–75 (1943)
21. Leibowitz, J., and Hestrin, S., *Biochem. J.*, **36**, 772–85 (1942)
22. Hanes, C. S., and Maskell, E. J., *Biochem. J.*, **36**, 76–79 (1942)
23. Green, A. A., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **142**, 447–48 (1942)
24. Hassid, W. Z., Cori, G. T., and McCready, R. M., *J. Biol. Chem.*, **148**, 89–96 (1943)
25. Astbury, W. T., Bell, F. O., and Hanes, C. S., *Nature*, **146**, 558 (1940)
26. Haworth, W. N., Heath, R. L., and Peat, S., *J. Chem. Soc.*, 55–58 (1942)
27. Hassid, W. Z., and McCready, R. M., *J. Am. Chem. Soc.*, **63**, 2171–73 (1941)
28. Colowick, S. P., and Sutherland, E. W., *J. Biol. Chem.*, **144**, 423–38 (1942)
29. Shapiro, B., and Wertheimer, E., *Biochem. J.*, **37**, 397–403 (1943)

30. Lutwak-Mann, C., and Mann, T., *Biochem. Z.*, **281**, 140–56 (1935)
31. Euler, H. v., and Adler, E., *Z. physiol. Chem.*, **235**, 122–53 (1935)
32. Dische, Z., *Naturwissenschaften*, **24**, 462 (1936)
33. Meyerhof, O., *Naturwissenschaften*, **23**, 850–51 (1935)
34. Ochoa, S., *J. Biol. Chem.*, **141**, 245–51 (1941)
35. Kleinzeller, A., *Biochem. J.*, **36**, 729–35 (1942)
36. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, **148**, 117–26 (1943)
37. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127–37 (1943)
38. Ostern, P., Guthke, J. A., and Terszakowec, J., *Z. physiol. Chem.*, **243**, 9–37 (1936)
39. Sakov, N. E., *Biochimia*, **6**, 163–77 (1941)
40. Lyubimova, M. N., and Engelhardt, V. A., *Biochimia*, **4**, 716–36 (1939)
41. Lyubimova, M. N., and Pevsner, D., *Biochimia*, **6**, 178–83 (1941)
42. Needham, J., Shen, S. C., Needham, D. M., and Lawrence, A. S. C., *Nature*, **147**, 766–68 (1941)
43. Needham, J., Kleinzeller A., Miall, M., Dainty, M., Needham, D. M., and Lawrence, A. S. C., *Nature*, **150**, 46–49 (1942)
44. Needham, D. M., *Biochem. J.*, **36**, 113–20 (1942)
45. Bailey, K., *Biochem. J.*, **36**, 121–39 (1942)
46. Kalckar, H. M., *Biol. Revs. Cambridge Phil. Soc.*, **17**, 28–45 (1942)
47. Mehl, J. W., and Sexton, E. L., *Proc. Soc. Exptl. Biol. Med.*, **52**, 38–40 (1943)
48. Marsland, D. A., and Brown, D. E. S., *J. Cellular Comp. Physiol.*, **20**, 295–305 (1942)
49. Engelhardt, V. A., and Lyubimova, M. N., *Biochimia*, **7**, 205–30 (1942)
50. Du Bois, K. P., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.*, **147**, 699–704 (1943)
51. Greville, G. D., and Lehmann, H., *Nature*, **152**, 81–82 (1943)
52. Du Bois, K. P., and Potter, V. R., *J. Biol. Chem.*, **148**, 451–52 (1943)
53. Du Bois, K. P., and Potter, V. R., *J. Biol. Chem.*, **150**, 185–95 (1943)
54. Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E., *Biochem. J.*, **34**, 1108–23 (1940)
55. Warburg, O., and Christian, W., *Biochem. Z.*, **311**, 209–10 (1942)
56. Warburg, O., and Christian, W., *Biochem. Z.*, **314**, 149–76 (1943)
57. Warburg, O., and Christian, W., *Biochem. Z.*, **314**, 399–408 (1943)
58. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71–92 (1943)
59. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **150**, 213–21 (1943)
60. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1942)
61. Utter, M. F., and Werkman, C. H., *J. Biol. Chem.*, **146**, 289–300 (1942)
62. Utter, M. F., and Werkman, C. H., *Biochem. J.*, **36**, 485–93 (1942)

63. Parnas, J. K., Ostern, P., and Mann, T., *Biochem. Z.*, **272**, 64–70 (1934)
64. Parnas, J. K., *Ergeb. Enzymforsch.*, **6**, 57–110 (1937)
65. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 675–82 (1942)
66. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149**, 529–41 (1943)
67. Morris, D. L., *J. Biol. Chem.*, **148**, 271–73 (1943)
68. Schiller, S., and Smith, O. W., *J. Clin. Endocrinol.*, **3**, 154–62 (1943)
69. Gülzow, M., and Hübner, H., *Klin. Wochschr.*, **21**, 706–9 (1942)
70. Dörle, M., *Klin. Wochschr.*, **21**, 53–56 (1942)
71. Soskin, S. Z., *J. Clin. Investigation*, **22**, 329–33 (1943)
72. Braun, A. E., *J. Biol. Chem.*, **145**, 197–99 (1942)
73. Seshagirirao, D., and Giri, K. V., *Proc. Indian Acad. Sci.*, **16B**, 190–204 (1942)
74. Kneen, E., and Sandstedt, R. M., *J. Am. Chem. Soc.*, **65**, 1247 (1943)
75. Little, J. E., and Caldwell, M. L., *J. Biol. Chem.*, **147**, 229–32 (1943)
76. Adams, M., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1359–68 (1943)
77. Wagreich, H., Halpert, W., and Hirschman, A., *J. Gen. Physiol.*, **26**, 479–80 (1943)
78. Fiegenbaum, J., *Biochem. J.*, **36**, 768–71 (1942)
79. Hestrin, S., Avineri-Shapiro, S., and Aschner, M., *Biochem. J.*, **37**, 450–56 (1943)
80. Hestrin, S., and Avineri-Shapiro, S., *Nature*, **152**, 49–50 (1943)
81. Hoffman, D. C., and Duran-Reynals, F., *Science*, **72**, 508 (1930)
82. McClean, D., *J. Path. Bact.*, **33**, 1045–70 (1930)
83. Duran-Reynals, F., *J. Exptl. Med.*, **58**, 161–81 (1933)
84. McClean, D., *J. Path. Bact.*, **42**, 477–512 (1936)
85. Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, **15**, 2790–97 (1931)
86. Boyland, F., and McClean, D., *J. Path. Bact.*, **41**, 553–65 (1935)
87. Duran-Reynals, F., *J. Exptl. Med.*, **69**, 69–81 (1939)
88. Claude, A., *J. Exptl. Med.*, **66**, 353–66 (1937)
89. Duran-Reynals, F., *Bact. Rev.*, **6**, 197–252 (1942)
90. McClean, D., *Biol. Revs. Cambridge Phil. Soc.*, **8**, 345–56 (1933)
91. Meyer, K., *Cold Spring Harbor Symposia Quant. Biol.* **6**, 91–102 (1938)
91a. Glick, D., *Ann. Rev. Biochem.*, **11**, 51–77 (1942)
92. Bacharach, A. L., Chance, M. R. A., and Middleton, T. R., *Biochem. J.*, **34**, 1464–71 (1940)
93. Humphrey, J. H., *Biochem. J.*, **37**, 177–81 (1943)
94. Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, **107**, 629–34 (1934)
95. Meyer, K., and Palmer, J. W., *Am. J. Ophthalmol.*, **19**, 859–65 (1936)

96. MADINAVEITIA, J., AND QUIBELL, T. H. H., *Biochem. J.*, **34**, 625–31 (1940)
97. MADINAVEITIA, J., AND QUIBELL, T. H. H., *Biochem. J.*, **35**, 456–60 (1941)
98. MCCLEAN, D., AND HALE, C. W., *Biochem. J.*, **35**, 159–83 (1941)
99. ROBERTSON, W. B., ROPES, M. W., AND BAUER, W., *J. Biol. Chem.*, **133**, 261–76 (1940)
100. MCCLEAN, D., *Biochem. J.*, **37**, 169–77 (1943)
101. CHAIN, E., AND DUTHIE, E. S., *Nature*, **144**, 977–78 (1939)
102. CHAIN, E., AND DUTHIE, E. S., *Brit. J. Exptl. Path.*, **21**, 324–38 (1940)
103. LYTHGOE, B., AND MADINAVEITIA, J., *Biochem. J.*, **37**, 6–9 (1943)
104. FAVILLI, G., AND BERGAMINI, L., *Biochem. Z.*, **313**, 243–49 (1942)
105. LUTWAK-MANN, C., *Biochem. J.*, **35**, 610–26 (1941)
106. MCCLEAN, D., *J. Path. Bact.*, **53**, 156–58 (1941)
107. MCCLEAN, D., *J. Path. Bact.*, **53**, 13–27 (1941)
108. MCCLEAN, D., *J. Path. Bact.*, **54**, 284–86 (1942)
109. MCCLEAN, D., AND ROWLANDS, J. W., *Nature*, **150**, 627 (1942)
110. FEKETE, E., AND DURAN-REYNALS, F., *Proc. Soc. Exptl. Biol. Med.*, **52**, 119–21 (1943)
111. MCCLEAN, D., ROGERS, H. J., AND WILLIAMS, B. W., *Lancet*, **1**, 355–60 (March 20, 1943)
112. MCCLEAN, D., AND ROGERS, H. J., *Lancet*, **1**, 707–8 (June 5, 1943)
113. FAVILLI, G., *Nature*, **145**, 866 (1940)
114. ROBERTSON, W. B., ROPES, M. W., AND BAUER, W., *Biochem. J.*, **35**, 903–8 (1941)
115. MADINAVEITIA, J., AND QUIBELL, T. H. H., *Biochem. J.*, **35**, 453–55 (1941)
116. PIRIE, A., *Brit. J. Exptl. Path.*, **23**, 277–81 (1943)
117. HUMPHREY, J. H., *Biochem. J.*, **37**, 460–63 (1943)
118. EVANS, E. A., SLOTIN, L., AND VENNESLAND, B., *J. Biol. Chem.*, **143**, 565 (1942)
119. EVANS, E. A., VENNESLAND, B., AND SLOTIN, L., *J. Biol. Chem.*, **147**, 771–84 (1943)
120. KRAMPITZ, L. O., WERKMAN, C. H., AND WOOD, H. G., *J. Biol. Chem.*, **147**, 243–53 (1943)
121. KALNITSKY, G., AND WERKMAN, C. H., *Arch. Biochem.*, **2**, 113–24 (1943)
122. KALNITSKY, G., WOOD, H. G., AND WERKMAN, C. H., *Arch. Biochem.*, **2**, 269–81 (1943)
123. UTTER, M. F., AND WERKMAN, C. H., *Arch. Biochem.*, **2**, 491–92 (1943)
124. CAJORI, F. A., *J. Biol. Chem.*, **143**, 357–61 (1942)
125. KENSLER, C. J., YOUNG, N. F., AND RHOADS, C. P., *J. Biol. Chem.*, **143**, 465–72 (1942)
126. LUTWAK-MANN, C., *Biochem. J.*, **36**, 706–28 (1942)
127. WESTENBRINK, H. G. K., AND VELDMAN, H., *Enzymologia*, **10**, 255–56 (1942)

128. Sealock, R. S., Nivermore, A. H., and Evans, C. A., *J. Am. Chem. Soc.*, **65**, 934 (1943)
129. Deutsch, H. F., and Hasler, A. D., *Proc. Soc. Exptl. Biol. Med.*, **53**, 63 (1943)
130. Davies, R., *Biochem. J.*, **37**, 230–38 (1943)
131. Green, D. E., Westerfield, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **145**, 69–84 (1942)
132. Woods, D. D., and Trim, A. R., *Biochem. J.*, **36**, 501–11 (1942)
133. Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **36**, 600–18 (1942)
134. Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **36**, 619–23 (1942)
135. Gale, E. F., *Bact. Rev.*, **7**, 139–73 (1943)
136. Gale, E. F., and Epps, H. M. R., *Nature*, **152**, 327–28 (1943)
137. Blaschko, H., *Biochem. J.*, **36**, 571–74 (1942)
138. Medes, G., and Floyd, N., *Biochem. J.*, **36**, 836–44 (1942)
139. Blaschko, H., *J. Physiol.*, **101**, 337–49 (1942)
140. Holtz, P., Credner, K., and Strubling, C., *Arch. exptl. Path. Pharmakol.*, **199**, 145–52 (1942)
141. Keilin, D., and Mann, T., *Nature*, **144**, 442 (1939)
142. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **136**, 425–34 (1940)
143. Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, **139**, 661–74 (1941)
144. Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, **140**, 445–51 (1941)
145. Scott, D. A., and Fisher, A. M., *J. Biol. Chem.*, **144**, 371–81 (1942)
146. Keilin, D., and Mann, T., *Nature* (In press)
147. Eirich, F. R., and Rideal, E. K., *Nature*, **146**, 541 (1940)
148. Petermann, M. L., and Hakala, N. V., *J. Biol. Chem.*, **145**, 701–5 (1942)
149. Main, E. R., and Locke, A., *J. Biol. Chem.*, **143**, 729–36 (1942)
150. Keilin, D., and Mann, T., *Nature*, **148**, 493–96 (1941)
151. Mann, T., and Keilin, D., *Nature*, **146**, 164–65 (1940)
152. Fegler, J., *Nature* (In press)
153. Jacobs, M. H., and Stewart, D. R., *J. Gen. Physiol.*, **25**, 539–52 (1942)
154. Free, A. F., Davies, D. F., and Myers, V. C., *J. Biol. Chem.*, **147**, 167–73 (1943)
155. Stevenson, S. S., *J. Clin. Investigation*, **22**, 403–9 (1943)
156. Kreps, E. M., and Chenykaeva, R., *Compt. Rend. Acad. Sci., U.R.S.S.*, **34**, 101–4 (1942)
157. Jaffé, W. G., *J. Biol. Chem.*, **149**, 1–7 (1943)
158. Berridge, N. J., *Nature*, **151**, 473–74 (1943)
159. Dziemian, A. J., *J. Cellular Comp. Physiol.*, **21**, 339–45 (1943)
160. Kies, M. W., and Schwimmer, S., *J. Biol. Chem.*, **145**, 685–91 (1942)
161. Huggins, C., and Neal, W., *J. Exptl. Med.*, **76**, 527–41 (1943)

162. Grossmann, M. I., Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, **138**, 676–82 (1943)

163. Pickford, G. E., *J. Exptl. Zoöl.*, **92**, 143–70 (1943)

164. Verbrugge, F., *J. Biol. Chem.*, **149**, 405–12 (1943)

165. Scott, E. M., and Sandstrom, W. M., *Arch. Biochem.*, **1**, 103–9 (1942)

166. Harper, A. A., and Raper, H. S., *J. Physiol.*, **102**, 115–25 (1943)

167. Walker, F., *Am. J. Physiol.*, **139**, 343–46 (1943)

168. Hind, H. G., *Biochem. J.*, **37**, 289–93 (1943)

169. Hind, H. G., *Biochem. J.*, **37**, 293–95 (1943)

170. Ferguson, J. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 243–45 (1943)

171. Petermann, M. L., *J. Biol. Chem.*, **144**, 607–16 (1942)

172. Woodward, G. E., and Reinhart, F. E., *J. Biol. Chem.*, **145**, 471–80 (1942)

173. Maver, M. E., and Thompson, J. W., *J. Natl. Cancer Inst.*, **3**, 383–87 (1943)

174. Rocha e Silva, M., and Anrade, O. S., *J. Biol. Chem.*, **149**, 9–17 (1943)

175. Turner, A. W., and Rodwell, A. W., *Australian J. Exptl. Biol. Med. Sci.*, **21**, 17–25 (1943)

176. Turner, A. W., and Rodwell, A. W., *Australian J. Exptl. Biol. Med. Sci.*, **21**, 27–36 (1943)

177. Haddock, J. N., and Thomas, L. E., *J. Biol. Chem.*, **144**, 691–95 (1942)

178. Page, I. H., and Helmer, O. M., *J. Exptl. Med.*, **71**, 29–42 (1940)

179. Plentl, A. A., and Page, I. H., *J. Biol. Chem.*, **147**, 135–41 (1943)

180. Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M., *J. Physiol.*, **98**, 283–98 (1940)

181. Munoz, J. M., Braun-Menendez, E., Fasciolo, J. C., and Leloir, L. F., *Am. J. Med. Sci.*, **200**, 608 (1940)

182. Edman, P., Euler, U. S. v., Jorpes, E., and Sjostrand, O. T., *J. Physiol.*, **101**, 284–88 (1942–43)

183. Friedman, M., and Kaplan, A., *J. Exptl. Med.*, **77**, 65–70 (1943)

184. Schales, O., *J. Am. Chem. Soc.*, **64**, 561–64 (1942)

185. Katz, Y. J., and Goldblatt, H., *J. Exptl. Med.*, **78**, 67–74 (1943)

186. Goldblatt, H., Katz, Y. J., Lewis, H. A., and Richardson, E., *J. Exptl. Med.*, **77**, 309–13 (1943)

187. Page, I. H., Helmer, O. M., Plentl, A. A., Kohlstaedt, K. G., and Corcoran, A. C., *Science*, **98**, 153–54 (1943)

188. Schales, O., Holden, M., and Schales, S. S., *Arch. Biochem.*, **2**, 67 (1943)

189. Plentl, A. A., Page, I. H., and Davies, W. W., *J. Biol. Chem.*, **147**, 143–53 (1943)

190. Fasciolo, J. C., Leloir, L. F., Munoz, J. M., and Braun-Menendez, E., *Rev. soc. argentin. biol.*, **16**, 643 (1940)

191. Croxatto, H., Croxatto, R., and Alliende, J., *Rev. soc. argentin. biol.*, **18**, 441–53 (1942)

192. Plentl, A. A., and Page, I. H., *J. Exptl. Med.*, **78**, 367–87 (1943)

193. Elson, L. A., *Nature,* **152,** 49 (1943)

194. Quastel, J. H., *Nature,* **152,** 215 (1943)

195. Turner, J. C., and Heath, F. K., *Nature,* **152,** 326 (1943)

196. Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.,* **147,** 443–62 (1943)

197. Mueller, G. C., and Rusch, H. P., *Cancer Research,* **3,** 113–16 (1943)

198. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.,* **147,** 99–108 (1943)

199. Rossi, A., *Arch. sci. biol.* (*Italy*), **28,** 40–59 (1942)

200. Edlbacher, S., and Viollier, G., *Z. physiol. Chem.,* **276,** 108–16 (1942)

201. Smythe, V. C., and Halliday, D., *J. Biol. Chem.,* **144,** 237–46 (1942)

202. Binkley, F., *J. Biol. Chem.,* **150,** 261–62 (1943)

203. Sizer, I. W., *J. Biol. Chem.,* **145,** 405–14 (1942)

204. Dounce, A. L., *J. Biol. Chem.,* **147,** 685–98 (1943)

205. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.,* **149,** 369–85 (1943)

206. Lawrie, N. R., *Biochem. J.,* **37,** 311–12 (1943)

207. Snyder, F. H., and Tweedie, W. R., *J. Biol. Chem.,* **146,** 639–47 (1942)

208. Drill, V. A., and Shaffer, C. B., *Gastroenterology,* **1,** 308–15 (1943)

209. Weil, L., and Russel, M. A., *J. Biol. Chem.,* **144,** 307–14 (1942)

210. Westenbrink, H. G. K., and Van Dorp, D. A., *Enzymologia,* **10,** 212–15 (1942)

211. Woodard, H. Q., *Cancer Research,* **3,** 159–63 (1943)

212. Gomori, G., *J. Biol. Chem.,* **148,** 139–49 (1943)

213. Ohlmeyer, P., *Naturwissenschaften,* **30,** 508 (1942)

214. Moog, F., *J. Cellular Comp. Physiol.,* **22,** 95–97 (1943)

215. Landow, H., Kabat, E. A., and Newman, W., *Arch. Neurol. Psychiat.,* **48,** 518–30 (1942)

216. Wolf, A., Kabat, E. A., and Newman, W., *Am. J. Path.,* **19,** 423–35 (1943)

217. Bourne, G., *J. Exptl. Physiol.,* **32,** 1–19 (1943)

218. Wilmer, H. A., *J. Exptl. Med.,* **78,** 225–29 (1942)

219. Moog, F., *Proc. Natl. Acad. Sci. U.S.,* **29,** 176–86 (1943)

220. Richter, D., and Godby Croft, P., *Biochem. J.,* **36,** 747–57 (1942)

221. Mendel, B., and Rudney, H., *Biochem. J.,* **37,** 59–63 (1943)

222. Mendel, B., and Mundell, D. B., *Biochem. J.,* **37,** 64–66 (1943)

223. Mendel, B., and Rudney, H., *Science,* **98,** 201 (1943)

224. Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.,* **37,** 473–76 (1943)

225. Eadie, G. S., *J. Biol. Chem.,* **146,** 85–93 (1942)

226. Strauss, O. H., and Goldstein, A., *J. Gen. Physiol.,* **26,** 559–85 (1943)

227. Torda, C., *Proc. Soc. Exptl. Biol. Med.,* **53,** 121 (1943)

228. Riechert, W., and Schmidt, E., *Arch. exptl. Path. Pharmakol.*, **199**, 66–73 (1942)

229. Riechert, W., and Schnarrenberger, C., *Arch. exptl. Path. Pharmakol.*, **200**, 225–34 (1942)

230. Bloch, H., *Helv. Chim. Acta*, **26**, 733–39 (1943)

231. Bullock, T. H., and Nachmansohn, D., *J. Cellular Comp. Physiol.*, **20**, 239–42 (1942)

232. Means, O. W., *J. Cellular Comp. Physiol.*, **20**, 319–24 (1942)

233. Armstrong, P. B., *J. Cellular Comp. Physiol.*, **20**, 47–53 (1942)

234. Armstrong, P. B., *J. Cellular Comp. Physiol.*, **22**, 1–19 (1943)

235. Sawyer, C. H., *J. Exptl. Zoöl.*, **92**, 1–11 (1943)

236. Sawyer, C. H., *J. Exptl. Zoöl.*, **92**, 11–29 (1943)

237. Feldberg, W., *J. Physiol.*, **101**, 432–45 (1942–43)

238. Kisch, B., Koster, H., and Strauss, E., *Exptl. Med. Surg.*, **1**, 51–65 (1943)

239. Koster, H., and Kisch, B., *Exptl. Med. Surg.*, **1**, 71–83 (1943)

240. Maegraith, B., and Findlay, G. M., *Nature*, **151**, 252 (1943)

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THE CHEMISTRY OF THE CARBOHYDRATES

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In the last two years the *Annual Review of Biochemistry* has had no review of the chemistry of polysaccharides. In Volume XII only the chemistry of sugars and their derivatives was treated. Inasmuch as important advances have been made during this period in the chemistry of polysaccharides, the present review will be confined entirely to this branch of carbohydrate chemistry.

In view of the great abundance of cellulose, starch, and glycogen in nature, and because of the great commercial importance of the first two, these polysaccharides have received much attention and have been studied intensively during the last two decades. Since the position joining the glucose units in these carbohydrates has been established as 1,4-, it was thought that this particular type of glycosidic linkage generally occurred when polysaccharides were elaborated in nature from their constituent monosaccharide residues. However, with the subsequent discovery of a great number of other polysaccharides, and with the accumulated knowledge of their structural configuration, it became evident that the 1,4-linkage is by no means the only one combining monosaccharide units in polysaccharides. Every possible combination of glycosidal linkage has now been found to exist, as may be illustrated by the following examples: yeast mannan (1) 1,2-; laminarin (2), or yeast polyglucose (3) 1,3-; galactocarolose (4) 1,5-; and glucosan from barley roots (5) 1,6-linkage.

Peat (6) divides the polysaccharides into two groups, the "simple" and the "complex," according to three different criteria. The first criterion is whether the polysaccharide consists of one kind or of different kinds of monosaccharide. Thus, starch, consisting entirely of glucose, is a "simple" polysaccharide, whereas arabic acid, made up of galactose, glucuronic acid, arabinose, and rhamnose, is a "complex" polysaccharide. The second criterion is whether there is uniformity or variance in the type of glycosidic linkage. For example, in cellulose and laminarin only β-1,4-linkages occur. On the other hand, yeast mannan, consisting entirely of mannopyranose units, contains in its structure three types of glycosidic linkages, the 1,2-, 1,3-, and 1,6-linkage. The third criterion is whether the polysaccharide

possesses a linear or branched structure. Cellulose, pectic acid, laminarin, and the amylose fraction of starch, possessing unbranched chains, are "simple," while gum arabic, damson or cherry gum, yeast mannan, and the amylopectin fraction of starch with a ramified structure, are "complex." A polysaccharide may be termed "complex" in more than one sense, as for example, the plant gums and the mucilages: they contain more than one type of monosaccharide unit and are also united by more than one kind of glycosidic linkage.

The expression "repeating unit" was introduced by E. L. Hirst and collaborators into the terminology of the chemistry of polysaccharides. It is used in connection with chemistry of starch and other polysaccharides as illustrated by the following example: an araban which exists in association with pectin (7) gives rise upon methylation and hydrolysis to three products in equimolecular proportions, namely, 2,3,5-trimethyl-*l*-arabinose, 2,3-dimethyl-*l*-arabinose, and 3-methyl-*l*-arabinose. Since the arabinose units in the polysaccharide have the furanose configuration, the possibility of linkage through the fourth carbon atoms of these units may be eliminated. A possible combination of the monosaccharide units that would form the araban and satisfy the experimental data is as follows: the trimethyl-*l*-arabofuranose is obviously a terminal unit and could be attached to the monomethyl derivative through position 2 or 5, this monomethyl derivative in turn being glycosidically linked through one of these linkages to position 1 of the dimethyl-*l*-arabinose. It is evident that this type of combination of three monosaccharide units, one of which is a monomethyl derivative, would produce a branched structure. Inasmuch as the molecular weight of the polysaccharide determined by osmotic pressure and viscosimetric measurements is many times greater than that of a combination of three arabinose residues, it is assumed that this branched low molecular weight structure exists as a "repeating unit" linked through primary valences to form a large polysaccharide molecule.

Starch

The recent interest in the chemistry of starch may be judged by the many papers that have appeared within the last few years on this subject. Noteworthy contributions have been made along the following lines: (*a*) combination of starch chains to form a ramified structure, (*b*) separation of whole starch into two or more components,

(*c*) the constitution of synthetic polysaccharides and their relation to natural starches, and (*d*) the nature of the starch iodine complex.

Constitution.—The Birmingham school, represented by Haworth and Hirst, considers the starch molecule to be made up of repeating units, each consisting of from twenty-four to thirty glucose residues, and combined by cross linkages to form a molecule of which the molecular weight is about 200,000 (8). The starch chains are combined in such a way that the free reducing group of a glucose unit of one chain is glucosidically linked with the sixth hydroxyl group of a glucose unit in an adjacent chain forming a ramified structure,[1] as shown in Fig. 1. They believe that the link binding the repeating

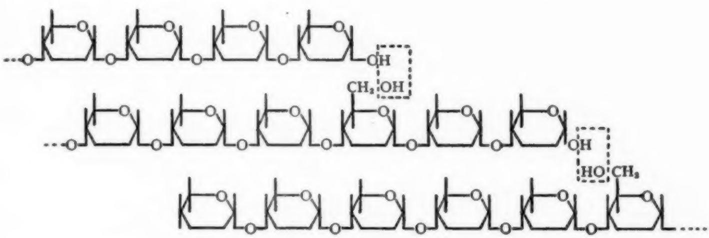

FIG. 1.—Starch (Bawn, Hirst & Young). [Cf. ref. 8.]

chains (units) in starch is a normal covalent bond (primary valence), such as is found, for example, in disaccharides. Evidence pertaining to the nature of the bond was adduced by Hirst & Young (9) from consideration of the reaction velocity and disaggregation process of methylated starches. The course of the disaggregation reaction of the latter was shown to be of the same order as those obtained on hydrolysis of compounds known to possess glycosidic linkages. They concluded that the repeating units of the starch molecule are held by normal covalent bonds, intermediate in strength, between that of a furanose linkage (as in sucrose) and a pyranose linkage (as in maltose).

[1] The same proportion of end group has been found in all the natural starches thus far examined, in spite of the considerable variations in the molecular weights of the different methyl derivatives. All the starches on which an end-group determination has been made are tabulated in a recent review of the chemistry of starch (10).

Hirst & Young showed that regardless of the method of preparation of the methyl derivatives, whether the starch is methylated directly in air, or in nitrogen, or whether the methylated starch is prepared through the acetate, and irrespective of the molecular weight of the methylated starch, the percentage of end group (tetramethylglucose) obtained on hydrolysis remains unchanged. Furthermore, it was possible to reduce the molecular weight of methylated starch by hydrolysis with aqueous methyl alcohol containing oxalic acid to a value of about 20,000 without changing the proportion of the nonreducing end group.

These results are contrary to the view of Richardson, Higginbotham & Farrow (11) who attributed the observed proportion of tetramethylglucose (end group) to random hydrolysis of long unbranched chains of similarly united residues during the preparation of the methyl derivatives from starch. In the opinion of Hirst & Young, if this were the case the proportion of end group should vary depending on the method of preparation of the methylated starch and should increase as the molecular weight of the methylated starch decreases. This, however, was not the case.

Freudenberg (12) bases his conception of starch structure on the screwlike model proposed by Hanes (13) shown in Fig. 2. The as-

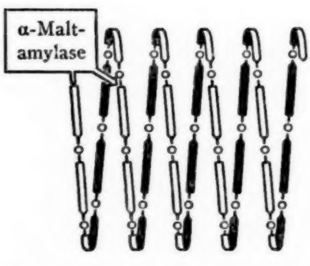

FIG. 2.—Hanes' hypothetical starch model. [Cf. ref. 13.]

sumption of such a model would explain the formation of closed ring, nonreducing Schardinger dextrins from starch, as well as the hydrolysis by α-amylase. Freudenberg postulates that the *Bacillus macerans* splits the starch to molecular chains containing five or six glucose residues. Because of the screwlike arrangement of the original spiral,

the first and last glucose units of these fragments are situated at a distance which is close enough to enable them to react and form rings of five or six glucose units. Thus, according to this author, the Schardinger dextrins are not preformed in the starch molecule, but are formed from the winding spiral of the starch chain in the process of enzymic hydrolysis. Indeed, Kerr (14) presents evidence indicating that the Schardinger dextrins are synthesized by the *B. macerans* enzyme from open chain dextrins.

Freudenberg *et al.* consider the α-dextrin to consist of a closed ring of five glucose units and the β-dextrin of six glucose units. Recent x-ray data, presented by French & Rundle (15), however, show that the α-dextrin (cyclohexaamylose) contains six glucose units, while the β-dextrin (cycloheptaamylose) contains seven glucose residues in the ring. X-ray diffraction data of butanol-precipitated amylose (16) as well as that of the amylose-iodine complex (17) also indicate that amylose possesses a hexagonal unit cell, the dimensions of which are in conformity with a helical structure. These dimensions are in agreement with those of a space-filling model of a helix with six glucose residues per turn.

According to Freudenberg (12), the screw-shaped starch molecule is considered to form rods of 15 to 20 Å thick, which are radially arranged in the concentric layers of the starch grain. Branching takes place in the spiral through linkage at the sixth hydroxyl group of the glucose units in the spiral. That branching occurs through the primary alcohol group is shown by the fact that the 2,3-dimethylglucose obtained, together with the 2,3,6-trimethylglucose and 2,3,4,6-tetramethylglucose, comes directly from the methylated starch (18, 19, 20). Thus, approximately every twentieth glucose unit has, in addition to the adjacent unit attached at the fourth carbon atom, another attached at the sixth position. From consideration of optical rotation data it is concluded that this linkage is of the α-type. Ahlborg & Myrbäck (21, 22) have also shown that α-glucosidic-1,6-linkages exist in dextrins obtained from starch. From these dextrins a saccharide was isolated, having a 1,6-linkage, believed to be isomaltose. Their data indicate that starch contains approximately one isomaltose for every fifteen to twenty maltose linkages. That the polymeric bonds are of the α-glucosidic type is demonstrated by Meyer & Bernfeld (23), inasmuch as they are hydrolyzed with α-glucosidase, but not with β-amylase.

Amylose, amylopectin, and synthetic starches.—It has been definitely established now that most natural starches contain at least two

components possessing a different constitution. Meyer *et al.* (24) separated maize and potato starch into two fractions, amylose and amylopectin. The amylose fraction, similar to the amyloamylose isolated by Samec (25) from potato starch by autoclaving and electrodialysis, was completely hydrolyzed with β-amylase to maltose. With unfractionated starch the enzymic hydrolysis ceases when approximately 60 per cent is converted into maltose. Meyer *et al.*, applying the end-group method to the two fractions of maize and potato starch, obtained a chain length of approximately 300 glucose units for amylose and 25 glucose units for amylopectin. Similar results were obtained by Hess & Krajnc (26) in the case of potato starch fractions. Since the molecular weight of methylated amylose determined by osmotic pressure measurements roughly agreed with that determined by the end-group method, Meyer *et al.* concluded that the amylose is not branched. The possibility that amylose is slightly branched is, however, not entirely excluded, inasmuch as Hess & Steurer (27) found that amylose with a chain length of 238 glucose units, based on end-group determination, showed a degree of polymerization of 650 glucose units by osmotic pressure measurements. In this case there are approximately three chains linked together to form an amylose molecule. Meyer and co-workers (28, 29) are of the opinion that maize starch consists of two components, each possessing a different molecular structure: amylose (10 to 20 per cent) a mixture of polymers with unbranched chains and having a molecular weight of from 10,000 to 60,000; and amylopectin (80 to 90 per cent) with greatly branched molecules, having a molecular weight of from 50,000 to 1,000,000.

McCready & Hassid (30) demonstrated that amylose exists in potato starch to the extent of about 20 per cent and that the intensity of the blue color produced with iodine is approximately six times that of amylopectin, and three times as intense as the color given by unfractionated starch. By examining the intensity of the blue color produced with iodine by mixtures of amylose and amylopectin, the relative proportion of each in the mixtures can be estimated. Hassid & McCready (31) substantiated the results of Meyer *et al.* (24) in regard to the chain length of the two potato starch fractions. The methylated amylose yielded on hydrolysis 0.3 per cent of tetramethylglucose, a value corresponding to a chain length of about 300 glucose units. On hydrolysis of the methylated amylopectin 4.7 per cent of tetramethylglucose was obtained. Considering the approximate mo-

lecular weights of the two fractions from viscosity measurements, these data indicate that amylose consists of long unbranched chains of about 300 glucopyranose units, whereas the amylopectin molecule is built of chains of 25 glucopyranose units, combined to form a ramified structure.

Bates, French & Rundle (32) developed a method for the quantitative estimation of amylose and amylopectin in various starches, based on the difference in the ability of the two fractions to bind iodine in complex formation. They found the amylose content of the various starches determined by this method to be as follows: tapioca 17, rice 17, banana 20.5, corn 21, potato 22, popcorn 23, wheat 24, sago 27, and lily bulb 34 per cent. It is of interest to note that waxy rice, waxy sorghum, waxy corn, and waxy barley starches are entirely devoid of amylose. Synthetic starch prepared by the action of potato phosphorylase on glucose-1-phosphate, in agreement with methylation studies, appeared to be essentially amylose. Preliminary results indicated that affinity for iodine of the starch components varied inversely with the degree of branching and directly with the length of the starch chains. The chains of the amylose component of any one starch were fairly homogeneous in length.

Meyer & Heinrich (33) compared the proportions of amylose and amylopectin in the different organs of the potato plant. They found that the starches isolated from green growing potato leaves and from tubers contained approximately 18 per cent of amylose and about 82 per cent of amylopectin. The amylose content of starch from the young sprouts on tubers was 46 per cent, a value rather unusually high for this component. These investigations were extended to a study of components of starches from sago, tapioca, peas, and waxy maize (34). Their results, in agreement with those of Bates *et al.* (32), indicated that waxy maize starch is devoid of amylose, and that the proportion of amylose and amylopectin in starches from different plant sources varies considerably.

Wolfrom *et al.* (35) applied the mercaptalation assay method to starch synthesized by the action of potato phosphorylase on glucose-1-phosphate. They hydrolyzed the synthetic starch with fuming hydrochloric acid at 0° in the presence of ethyl mercaptan. The mercaptalated mixtures, resulting from the reaction of the ethyl mercaptan and the released reducing groups by hydrolysis, were isolated as their acetates at various time intervals. The sulfur analytical data and the course of the hydrolytic reaction (without mercaptalation) were in-

terpreted by these authors as showing an average degree of polymerization of synthetic starch similar to that of natural starch. By graphic analysis a value of 32 ± 1 glucose units was obtained for the initial average degree of polymerization of the synthetic potato starch. On this basis, Wolfrom *et al.* eliminate the long chain structure for synthetic starch and conclude that it has the same type of bond as the repeating unit in natural starch. However, there is no reason to suppose, on theoretical grounds, that the "average degree of polymerization" obtained by this method represents a definite structural unit in the starch molecule. Indeed, it may be observed that the mercaptalation method gave no consistent results when applied to natural starch. An average degree of polymerization of 20 ± 4 was obtained with unmethylated potato starch (36); with methylated starch (37) the degree of polymerization was 150. The results of 20 ± 4 glucose units reported in one case by Wolfrom *et al.* may therefore be considered as a chance agreement with the values obtained by Haworth's end-group method for natural starches.

By means of selective precipitation with butanol, Schoch (38) isolated a fraction in 22 per cent yield from either corn or potato starch in "unique spherocrystalline form." These crystals are considered by Schoch to be formed as a result of the formation of an addition compound with the butanol. No "end-group" determination has yet been made on this crystalline fraction. According to Bear (39), the butanol precipitated crystalline starch fraction, similar to the synthetic polysaccharides, gives a V-type x-ray pattern. As in potato amylose and the synthetic polysaccharides, this crystalline fraction has a high conversion limit to maltose with β-amylose (40) and a great tendency to adsorb iodine, and is therefore considered the same as amylose (32). Wilson, Schoch & Hudson's (41) observation that the butanol-precipitated fractions from corn and potato starches give high yields (approximately 70 per cent) of Schardinger dextrins, whereas the non-precipitated fractions give low yields (approximately 49 per cent) of these dextrins, lends further support to this view. According to these workers, this suggests a relatively uniform molecular configuration for the precipitated fraction and a more complex structure for the nonprecipitated fraction. Kerr & Severson (40), applying Schoch's butanol precipitation method to a hot water extract of corn starch, obtained a product in the form of single minute crystals, which is also considered to be amylose, having an unbranched structure (32). Kerr *et al.* (14, 40, 42) reported the existence of a third

starch component (γ-amylose) isolated as an insoluble precipitate resulting from the conversion of corn starch with β-amylase or with *B. macerans* amylase. Wilson, Schoch & Hudson (41) attribute this insoluble fraction to the presence of fatty material and incomplete dispersion of the starch. Defatted starch, properly dispersed before submitting to enzyme action, produces a negligible amount of such insoluble material.

Pacsu & Mullen (43) reported a method of starch fractionation by selective adsorption on cotton. However, they do not show proof of the purity of the amylose obtained by this method. Meyer & Fuld (44) found that the amylose prepared from potato starch by Wiegel (45) by hot dilute alcohol extraction was a mixture of amylose and amylopectin.

Foster & Hixon (46) show that the viscosity limits of amylose components from various starches fall in an inverse order to the potentials at which they take up iodine from solution. This fact, they believe, confirms the assumption that the potential is a function of the molecular weight of the amylose. An examination of the viscosity-concentration relationships of the amyloses indicates that the amyloses have a linear structure. Synthetic starch, however, appears to be anomalous in its behavior both with respect to its iodine-titration curve and the viscosity-concentration relationship of its solutions.

Levine, Foster & Hixon (47) studied the dextrins isolated from corn syrup. By repeated methanol fractionation they obtained fractions ranging in mean molecular weight (calculated from iodine-reducing values) from less than two to twenty-six glucose units. Methylation data indicated that the chains are essentially unbranched, inasmuch as only a negligible quantity of dimethylglucose, as compared with that of tetramethylglucose, was found in the hydrolysis products of the methylated dextrins. Levine *et al.*, in evaluating their methylation data, express the opinion that the extent of branching can be more accurately estimated if, instead of tetramethylglucose, the quantity of dimethylglucose is determined. It should be pointed out that Freudenberg & Boppel (19) demonstrated that about 40 per cent of the dimethylglucose obtained on hydrolysis of methylated starch is a degradation product, formed from 2,3,6-trimethylglucose during hydrolysis. The tetramethylglucose is shown to be unaffected. It is therefore obvious that no reliance can be placed on the determination of dimethylglucose as an assay for evaluation of the extent of branching.

Caldwell & Hixon (48), comparing the physical properties of the

limit dextrins from waxy and ordinary corn starch by digestion with β-amylase, found "a close similarity rather than a chemical difference between them." The quantity of dimethylglucose obtained by hydrolysis of methylated corn starch was 0.93 per cent and that from its limit dextrin 0.67 per cent. They consider the difference (26 per cent) as insignificant and conclude that, if branching exists in the original starch, the linkages involved are hydrolyzed by β-amylase. Inasmuch as these authors base their conclusion on dimethylglucose rather than tetramethylglucose data, their results cannot be accepted as significant for the reason previously stated.

Synthetic starch, prepared by Hanes (49) through the action of potato phosphorylase on glucose-1-phosphate (Cori ester), is similar to potato amylose in being completely hydrolyzed with β-amylase to maltose. Furthermore, the synthetic starch closely resembles the amylose in solubility, in tendency to retrograde from solution, and in the intensity of the blue color resulting from the addition of iodine. Data obtained by the end-group method (50, 51) reveal that synthetic starch, similar to amylose, is made up of long chains of glucopyranose units with little or no branching. It was demonstrated by Hassid, Cori & McCready (52) that the polysaccharide obtained through the action of crystalline muscle phosphorylase on glucose-1-phosphate *in vitro* is also similar in its properties and molecular constitution to the amylose fraction from potato starch. This synthetic muscle starch, unlike natural glycogen or starch, is made up of unbranched chains of about 200 glucose units.

There appears to be a definite correlation among certain of the properties of different starch-like polysaccharides and their molecular constitutions. Amylose from natural starch and the synthetic polysaccharides synthesized *in vitro* by the potato and muscle phosphorylases have the following properties in common: (*a*) sparing solubility in water, (*b*) intense color production with iodine, (*c*) production of a sharp x-ray V-diffraction pattern (39), and (*d*) complete hydrolysis with β-amylase to maltose. These properties are identified with an unbranched long chain structure. On the other hand, the more soluble amylopectin and animal glycogen produce a comparatively less intense color with iodine, give a diffuse x-ray pattern, and are incompletely hydrolyzed with β-amylase. These substances are recognized as having a branched and relatively short-chain structure.

The difference in behavior of β-amylase toward amylose, or the synthetic polysaccharides and amylopectin, or glycogen may be ex-

plained on the basis of their difference in chemical constitution (23, 31). β-amylase attacks the nonreducing ends of the polysaccharides, splitting off successive maltose fragments until it encounters a modification in structure. Since branching in starch and glycogen occurs on the sixth carbon atom of some of the glucose units in the chains, it is assumed that these 1,6-linkages are probably responsible for stopping the hydrolysis at or near the points of branching. With long-chain polysaccharides having a nonbranched structure and therefore no such linkages, the hydrolysis by β-amylase continues until the whole molecule is degraded to maltose.

Starch-iodine color and miscellaneous. — Freudenberg (12) explains the blue starch-iodine color on the basis of a helical structure of starch. When treated with iodine, the iodine molecules are deposited within the spirals, the spaces of which are of the exact size to allow the iodine to enter. The association formed between the iodine and the starch spirals affects its absorption bands, thus producing the blue color. Meyer & Bernfeld (53) object to this explanation on the grounds that some substances known not to have a spiral arrangement, such as methylated cellulose, or cellulose which has been swollen with zinc chloride, also give a blue color with iodine. According to these authors, the explanation of the blue iodine color resides in the colloidal structure of these compounds. They note that certain colloidal suspensions and finely divided precipitates also give colors with iodine similar to starch. None of these substances gives the color reaction with iodine either in the state of molecularly dispersed solutions or in crystalline state; the color occurs only in the case of colloidal precipitates having greatly distorted lattices. The iodine molecules are deposited in the fissures of these particles, forming a complex which results in a change of the light absorption of the iodine. Similarly, in the case of starch, the iodine is deposited in the supermolecular aggregates or micelles which produces an effect on the absorption bands of the iodine.

Rundle *et al.* (54, 55) claim that, since no one model is capable of explaining the many known colored iodine addition products other than starch, Meyer's objection to the helical starch model is not serious. These workers accept Freudenberg's starch model and present further evidence to strengthen this view by showing that starch-iodine solutions exhibit dichroism of flow; the light with its electric vector parallel to the flow lines is more strongly absorbed than light with its electric vector normal to the flow lines. The dichroism of flow requires

that the long axes of the iodine molecules in the complex be parallel to the long axis of the starch-iodine complex. They believe that the optical properties of starch iodine and starch-iodine fractions may be best interpreted in terms of helical starch chains.

Schoch (56) showed that the fatty acids, formerly believed by Taylor and associates to be esterified with starch, are merely adsorbed impurities. He demonstrated that the fatty material in corn, rice, and wheat starches can be removed without hydrolytic degradation by extraction with suitable water-miscible fat solvents such as methanol or 80 per cent dioxane. Lehrman (57), in his study on the nature of the fatty acids associated with starch, arrived at a similar conclusion.

Schoch's (56) results indicate that, while the greatest proportion of the phosphorus material can be extracted with hydrophilic fat solvents from corn, wheat, and rice as lipid material, the same treatment removes only a very small fraction of the phosphorus in potato starch. This phosphorus is probably esterified with the carbohydrate. These results are in agreement with those of Posternak (58) who found that, whereas in the starches stored in tubers and rhizomes the phosphorus is combined in ester form with starch, in the cereals the phosphorus is not directly linked to the starch but rather is present in the form of lecithins. Schoch recommends that prior to the use of the common cereal starches for any fundamental studies, they should be freed from lipid material by successive extraction with hot 85 per cent methanol.

Lampitt, Fuller & Goldenberg (59) report the results of a comprehensive investigation on the effect of prolonged dry grinding upon the physical properties and molecular size of wheat starch. They found that the following major changes take place during the grinding process: (*a*) the granules lose their organized "micro-structure" and become permeable to cold water; (*b*) the proportion of the ground starch extractable by cold water increases with the progress of grinding until, after 2,000 hours' grinding, nearly all the starch becomes soluble; (*c*) the phosphorus-poor fraction of the wheat starch is broken down by grinding preferentially to the phosphorus-rich fraction, but both are eventually reduced to a form soluble in cold water. The starch soluble in cold water is disrupted through the gradual break-up of the macromolecules by rupture of the lateral linkages which hold together the repeating units of twenty-four to thirty anhydroglucose residues (see Fig. 1).

Bear & French (60) studied the x-ray diffraction patterns of several starches obtained from various plant sources. Their data indicate

that the diffractions are those of a single major component and that in potato starch and corn starch there are two maltose residues per unit cell.

Glycogen

Several additional specimens of glycogen from different sources (dogfish muscle, *Helix pomatia,* and livers of dogfish, haddock, and hake) were examined by the end-group method (61, 62). The data obtained were consistent with previous results (63), which led to the conclusion that glycogen possesses a branched structure, the molecule consisting of repeating chains of twelve or eighteen glucopyranose units.[2] Haworth and co-workers (61) now consider the repeating units to be held together by primary valences.

A polysaccharide isolated from a higher plant, the seed of *Zea mays,* possessed properties commonly associated with glycogen (64). An examination of this carbohydrate by the end-group method showed that its structure is similar to that of glycogen from animal sources (65).

A more precise representation of the structure of glycogen was formulated by Meyer & Fuld (66). These workers envisage multiple branching rather than single branching, as conceived by Haworth, Hirst & Isherwood (63). They find that methylated glycogen, assayed by the end-group method, has one terminal glucose unit per eleven glucose residues (9 per cent tetramethylglucose), and that glycogen is degraded by β-amylase to maltose to the extent of 47 per cent. The 53 per cent of the residual dextrin resisting β-amylase contained 18 per cent tetramethylglucose, which is equivalent to one terminal group per every 5.5 glucose units. They believe this to indicate that the outer branches of the glycogen molecule, which is attacked by the enzyme, consist of six or seven glucose residues in α-1,4-glucosidic binding; 5.5 of these residues are split off by β-amylase, whereas one or two remain at the branching point, furnishing the terminal groups of the residual dextrin. Only short chains of an average of three glucose residues with free 2,3,6-OH groups are situated between these glucose residues whose 6-position is occupied by a branch. This conception of a ramified glycogen structure is represented in Fig. 3 (p. 72).

[2] The glycogens from various sources on which end-group determinations have been made are tabulated in a recent review (10).

A similar view of multiple branching is held by Meyer (23) regarding the structure of amylopectin, with the difference that the outside branches consist of fifteen to eighteen glucose units, and the inside parts of the chain, between branch positions, of about eight or nine units. Since both the outside and inside branches in glycogen are shorter than in amylopectin, the glycogen lends itself to the building of a more compact molecule. The glycogen molecule is considered spherical in shape; however, viscosity-concentration relationship studies indicate a deviation from the completely spherical form (67).

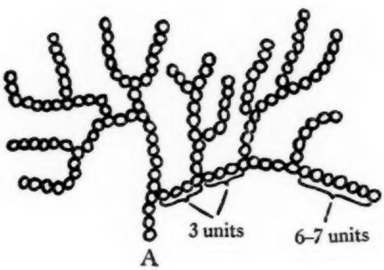

FIG. 3.—Glycogen (Meyer, A = aldehydic end, o = glucose units). [Cf. refs. 66, 67.]

The molecular weight of glycogen determined by osmotic pressure measurements is in the region of 1,000,000 to 2,000,000 (68). According to Meyer's (67) observations, undegraded glycogen from various origins has a molecular weight in excess of 4,000,000.

CELLULOSE

Whereas the yield of end-group from methylated starch remains the same regardless of whether the methylation process is carried out in an atmosphere of air or nitrogen, in the case of cellulose these conditions exert a marked influence. Hess & Neumann (69) demonstrated that when oxygen is carefully excluded during methylation of cellulose by displacing the air with nitrogen, no tetramethylglucose is present among its hydrolysis products. The end groups which they found in experiments where methylation was carried out in the presence of oxygen were attributed to an oxidative degradation caused by

alkali in the presence of air. Haworth and collaborators (70, 71) reinvestigated the problem of disaggregation of cellulose during the methylation process in air and in nitrogen and made the additional observation that, while no end groups are observed when cellulose is methylated in the presence of nitrogen, the cellulose is nevertheless progressively broken down to smaller particles when repeatedly methylated. Successive methylation in air causes degradation of cellulose to smaller-sized particles, the degradation being reflected in the results obtained by viscosimetric and osmotic pressure measurements and also by the end-group method. On the other hand, if air is excluded during the process of repeated methylation, the molecular size of the cellulose is similarly diminished, as measured by the same physical methods, but the tetramethylglucose is entirely absent from the hydrolysis products. The diminution of the particle size, however, proceeds at a much slower rate when the methylation is conducted in nitrogen than when in air. Thus, methylated cellulose, prepared by sixteen methylations in air (70, 71) was shown by osmotic pressure measurements to consist of 43 glucose units (by the end-group method, 66 glucose units); whereas when air was excluded the product of fifteen methylations gave a molecular weight of approximately 450 glucose units determined by the osmometric method (only negligible amounts of tetramethylglucose were found by the end-group method). However, when methylated in an atmosphere of nitrogen, no ultimate disruption of cellulose occurred. The molecular weight tended toward a minimal value of approximately 200 glucose units, which was reached after twenty-five to thirty methylations.

The distinction between obtaining a methyl cellulose containing tetramethylglucose when methylated in the presence of air, and that of a methylated product devoid of end groups when air is excluded applies only to cellulose which is directly methylated. When methylated cellulose is prepared from cellulose which had been previously acetylated, the atmosphere in which the methylation is conducted appears immaterial. Whether the methylation process is carried out in nitrogen or in air, the products from the acetate invariably contain end groups corresponding to an average chain length of approximately 200 glucose units. The fact that the size of methylated cellulose particles diminishes with successive methylation in air as well as in nitrogen, indicates that the rupture of glucosidic linkages takes place at definite points along the chains. Haworth (72) accounts for the absence of end groups in cellulose methylated with methyl sulfate and

alkali in nitrogen, notwithstanding the fact that degradation of the methylated cellulose takes place, by the assumption that molecular degradation is followed by immediate formation of loops. The changes that take place during methylation are shown graphically in Fig. 4.

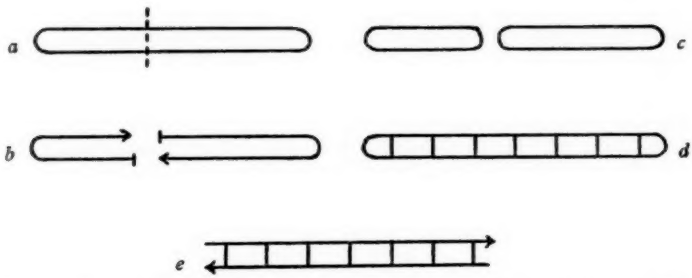

FIG. 4.—Degradation of methylated cellulose in the presence and absence of air (Haworth). [Cf. ref. 72.]

Methylated cellulose of high molecular weight is represented by a loop, Fig. 4(*a*). Further methylation leads to simultaneous rupture at two points as shown by the dotted line in Fig. 4(*a*) and results in two open-chain structures, Fig. 4(*b*). In the absence of oxygen the exposed ends at the points of rupture recombine and form smaller loops, Fig. 4(*c*), which produce no end groups. Oxygen, however, appears to inhibit the recombination of the ends of these fragments, Fig. 4(*b*), inasmuch as it is observed that methylated cellulose obtained when the methylation is conducted in air contains an end group. In order that formation of loops could take place it is assumed that the ends of each fragment, represented by Fig. 4(*b*), are situated in close proximity one to another and remain in the same position as at the time of rupture. Haworth (72) suggests that the alignment, whereby the ends are held in static position, is maintained by lateral cross-linkages, as shown in Fig. 4(*d*). This hypothesis implies that native cellulose consists of long chains of glucose residues joined by 1,4-β-glucosidic linkages and that these chains are oriented with respect to each other in such a way that alternate chains are aligned with the potential reducing groups pointing in opposite directions, Fig. 4(*e*). The chains are held by lateral secondary bonds which serve to maintain the alignment and occur at intervals of twenty-five

to thirty glucose units. Ring closure at each end of a pair of such chains would produce a looped structure, shown in Fig. 4(*d*).

Haworth *et al.* (73, 74) studied the cellulose product obtained by soaking cotton in 5 per cent hydrochloric acid. The hydrocellulose thus prepared did not contain any uronic acid residues but retained its fibrous structure. This hydrocellulose was further separated into a powdered and a fibrous fraction. End-group assay showed that the original hydrocellulose had an average chain length of 120 glucose units. The chain length of the powder form, determined by oxidation with iodine, corresponded to seventy glucose units. Hydrocellulose thus appears to be a cellulose partially degraded through rupture of some glucosidic linkages in the chains under the influence of acid during preparation. It is therefore considered to constitute fragments of the original cellulose chain molecules, the essential structure of these fragments being the same as in the parent cellulose. The difference between the powdered and fibrous fractions is that in the former the hydrolytic cleavage proceeded farther than in the latter. It appears, from the study of solubilities of various hydrocellulose fractions in sodium hydroxide, that their solubilities are inversely proportional to their chain lengths.

Oxycellulose, prepared by oxidation of cotton linters in cold 0.25 *N* acid permanganate (75) retained the fibrous structure of cellulose, but was noted by its high reducing value, extreme sensitivity to alkali, and uronic acid content. Fifty per cent of the oxycellulose dissolved in 0.25 *N* solution of alkali and the dissolved portion underwent decomposition of the type suffered by oligosaccharides in the presence of alkali. The alkali insoluble residue of oxycellulose did not show the characteristic properties of the original oxycellulose and was practically indistinguishable from hydrocellulose prepared by treating cellulose with dilute acid. It acetylated and methylated with ease and its molecular size, determined by the end-group method, corresponded to 90 glucose units.

Purves *et al.* (75*a*) have studied the distribution of the acetyl groups in technical cellulose acetate, having an average of 2.44 out of a possible 3 acetyl groups per glucose unit. They esterified the remaining 0.56 hydroxyl groups in the incompletely acetylated cellulose with *p*-toluenesulfonyl (tosyl) chloride and determined the rates at which the hydroxyls in the three different positions were tosylated. The tosyl group on position 6 could be determined by direct reaction with sodium iodide, but positions 2 and 3 were estimated by

mathematical analysis of their rates of esterification. The rate constants for tosylation of the unsubstituted hydroxyl groups in the cellulose acetate were found to be in the ratio of 23.4 : 2.16 : 0.106 for positions 6, 2, and 3, respectively. When the free hydroxyl groups of incompletely ethylated ethylcellulose were similarly substituted by tosyl groups, a tosylation rate ratio of 15: 2.3 : 0.07 was obtained for positions 6, 2, and 3 of the anhydroglucose units. This ratio is approximately the same as demonstrated in the case of cellulose acetate. It may, therefore, be concluded that in esterification and alkylation reactions the hydroxyls in position 6 (primary alcohol group) of the glucose units in the cellulose chain possess the greatest reactivity, while the hydroxyls in position 3 react most sluggishly.

Polysaccharides Containing Galactose

Arabo-galactan. — The arabo-galactan, or so-called ε-galactan, known to exist in larch woods and of interest in connection with the chemistry of wood formation, has been further studied by Wise and co-workers (76). Hydrolyzed specimens of arabo-galactan from different species of larch wood yielded arabinose and galactose in an approximate ratio of 1 to 6 moles. Systematic fractionation of the ester derivatives of this carbohydrate from various solvent mixtures produced fractions of similar acyl content but of variable optical activity, reducing value, specific viscosity, and araban content. These authors concluded that the ester derivatives were nonhomogeneous and that the arabo-galactan was probably also a mixture of a galactan and an araban. Hirst *et al.* (77) arrived at a similar conclusion, inasmuch as the methyl derivative of arabinose-free galactan obtained by preferential hydrolysis of the araban with dilute acid was identical to the methylated galactan obtained by separation of the latter from a mixture of methylated galactan and methylated araban produced by methylation of the original arabo-galactan. The araban consisted of arabofuranose[3] units, whereas the galactan was composed of

[3] According to Hirst (78), the existence in close association of two polysaccharides, araban and galactan, containing monosaccharide units of different ring structure is not consistent with the frequently advanced "decarboxylation theory." This theory claims that pentoses originate intermolecularly through decarboxylation of uronic acids ultimately derived by oxidation of hexoses. Thus, for example, *d*-xylose in the polysaccharide xylan found in close association with cellulose may be derived from *d*-glucose of the latter; similarly *l*-arabinose in araban

β-linked galactopyranose units. After methylation and hydrolysis, the galactan produced an equimolecular mixture of tetramethyl-, 2,4-dimethyl-, and a trimethylgalactopyranose, indicating that the polygalactose consisted of branched-chain repeating units.

Mosimann & Svedberg's (79) results also indicate that the arabo-galactan is a mixture of two polysaccharides. Using the ultracentrifuge, they obtained sedimentation diagrams showing that the centrifuged substance contained two components of different molecular weights, approximately 16,000 and 100,000.

Contrary to the results obtained by the previous investigators, White (80) presents evidence that the arabo-galactan from western larch is not a mixture of two polysaccharides, but is one chemical entity joined by oxygen linkages through the reducing carbon atoms of the pentose units to sixth positions of galactose residues. On methylation and complete hydrolysis of the polysaccharide, 2,4-dimethyl-*d*-galactopyranose (3 moles), 2,3,4-trimethyl-*d*-galactopyranose (1 mole), 2,3,4,6-tetramethyl-*d*-galactopyranose (2 moles), and 2,3,5-trimethyl-*l*-arabofuranose (1 mole) were obtained. Partial hydrolysis of the methylated arabo-galactan also yielded a variety of fission fragments, including octamethyl- and heptamethyl-6-*d*-galactosidogalactose, and a residue comprising mainly 2,4-dimethylgalactose anhydride units. The methylation data indicate a main chain of 1,6-linked galactopyranose units previously proposed by Hirst and co-workers (77). Each unit of the primary chain is substituted in position 3 by a secondary chain of three 1,3-linked galactose residues, each in turn substituted at the 6-position by terminal units. The latter are, respectively, *d*-galactose and the radicals R, *d*-galactose and *l*-arabinose, whose relative position is not known. The following tentative diagrammatic Formula I (p. 78) representing the repeating unit of the arabo-galactan, is proposed by White.

Agar.—Percival & Somerville (81), using the methylation pro-

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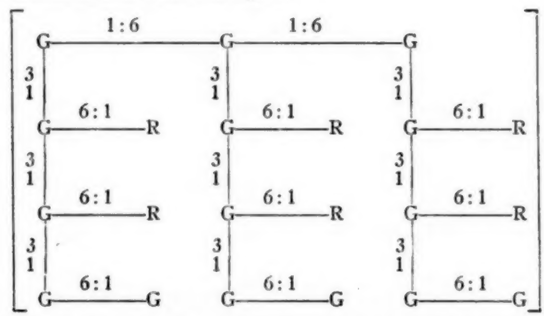
may be derived from *d*-galactose of galactan. Since the arabinose units in araban have the furanose configuration, they could not have been derived directly from galactose by oxidation and subsequent decarboxylation. Such a transformation should yield arabopyranose units. Hirst is of the opinion that the mechanism of decarboxylation is not involved in the production of pentose sugars.

As far as it is known, whenever *l*-arabinose is encountered in nature in combined form, as in the plant gums or the arabans associated with pectic substances (7, 78), it always exists in the furanose form. On the other hand, combined xylose favors the pyranose form.

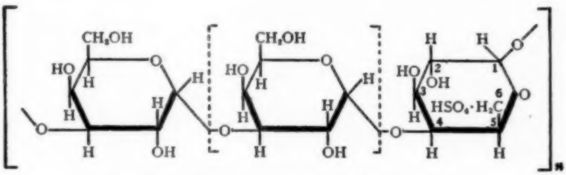
cedure, were first to establish the fundamental features of the structure of agar. They showed that the major part of the agar molecule consists of *d*-galactose residues united by 1,3-glycosidic linkages chiefly of the β-type. The sulfuric acid group esterified with galactose is hydrolyzed during acetylation or methylation, since no sulfur can be detected in agar acetate or methylated agar. Pirie (82) made the interesting observation that *l*-galactose as well as *d*-galactose was present among the acetolysis products of agar. That *l*-galactose is an integral part of this polysaccharide was later definitely established from the fact that a derivative of *l*-galactose was found among the hydrolysis products of methylated agar (83). Araki (84) also reports the isolation of a disaccharide from the hydrolysis products of methylated agar which is apparently pentamethyl-*d*-galactosido-3,6-anhydromethyl-*l*-galactoside, the *d*- and *l*-galactose derivatives probably united through the first and fourth carbon atoms. It was further shown that the methylated *l*-galactose derivative contained a 3,6-anhydro (or hydrofuranol) ring. But, inasmuch as Duff & Percival (85) demonstrated that alkaline hydrolysis of methylgalactoside-6-sulfate leads to the formation of 3,6-anhydromethylgalactoside, it is assumed the 3,6-anhydro form of *l*-galactose obtained from agar is not an original constituent, but a residue formed in the course of hydrolysis of *l*-galactose-6-sulfuric acid ester in agar.



I. Arabo-galactan. (G = *d*-galactopyranose residue; R = *d*-galactopyranose or *l*-arabofuranose, but not both; stroke indicates a glycosidic link; numerals represent points of attachment of glycosidic linkages. For convenience the reviewer has chosen the diagrammatic presentation of the more complex polysaccharides.)

Jones & Peat (86) found among the hydrolysis products of methylated agar some carboxylic acid groups, identified as 2,5-dimethyl-

3,6-anhydro-*l*-galactonic acid. This acid is believed to form by air oxidation from the terminal *l*-galactose residue when acid conditions precede the preparation of the methylated agar. According to these authors, the agar is the sulfuric acid ester of a linear polygalactose in which the repeating unit is composed of nine *d*-galactopyranose residues terminated at the reducing end by one residue of *l*-galactose. The *d*-galactose units are mutually combined by 1,3-glycosidic linkages, but the *l*-galactose residue is attached to the chain through position 4. The *l*-galactose is esterified at the sixth carbon atom with sulfuric acid. According to Jones & Peat, the constitution of agar is best represented by Formula II.



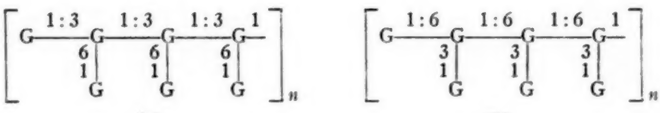
II. Agar (Jones and Peat). [Cf. ref. 86.]

The results of Percival & Thomson (87) regarding the relative proportion of the hydrolysis products of the methylated agar derivatives are somewhat at variance with those obtained by Jones & Peat. Percival & Thomson point out that the methoxyl content for a model of methylated agar on the line suggested by Jones & Peat should be 42 per cent, whereas the highest recorded value is not greater than 35 per cent for a representative sample. They are, therefore, of the opinion that the structure of agar as presented by Formula II is oversimplified.

Buchanan, Percival & Percival (88) have shown that the polysaccharide from carragheen moss, similar to agar, contains galactose residues joined by 1,3-linkages. However, the sulfuric ester group is attached to carbon atom 4 in galactose and not to position 6 as in agar. The galactosidal linkages combining the residues appear to be of the α-type. This takes no account of the mode of union of the unidentified portion of the molecule. It is of interest to note that the 1,3-glycosidal linkage is prevalent in galactose polysaccharides.

Snail galactogen.—This carbohydrate occurs together with glycogen in the albumin glands of the snail, *Helix pomatia*. According to

Baldwin & Bell (89), hydrolysis of the methylated galactogen produced a mixture of 2,3,4,6-tetramethyl-*d*-galactopyranose and 2,4-dimethyl-*d*-galactopyranose, but no trimethylgalactose could be detected. These authors (90) also demonstrated that part of the galactose in galactogen exists in the form of the *l*-isomer, inasmuch as *dl*-galacto-2-benziminazole was prepared from the hydrolysis products of the polysaccharide. This is the first case on record showing that *l*-galactose exists in a material of animal origin. Since trimethylgalactose is absent in the hydrolysis products of the methylated galactogen, the repeating unit cannot be represented by an unbranched, but rather must be represented by a highly branched, chain. The repeating unit probably consists of seven galactopyranose units. The relative proportion of tetra- and dimethylgalactose indicates two possible structures for galactogen, III*a* or III*b*.



(*a*) (*b*)

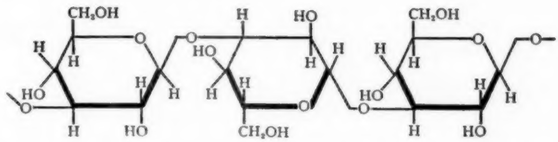
III. Snail galactogen. (Two possible arrangements of the repeating unit; G = galactopyranose residue.) [Cf. ref. 90.]

Optical rotation data point to a 6:1 ratio of *d*- to *l*-galactose in galactogen. The exact position of the *l*-galactose is unknown, but the indication is that one of the four terminal galactose residues in the repeating unit is *l*-galactose. The colloidal properties of galactogen suggest that it is very likely a large molecule made up of a great number of repeating units, each consisting of seven galactose residues, one of which is reducing. The repeating units are probably joined through the reducing galactose residue in one unit and some hydroxyl group of a galactose residue of another unit. Such linkage would account for the nonreducing properties of the galactogen.

Other Polysaccharides

Laminarin.—The constitution of the polyglucose, laminarin, known to occur in *Laminaria cloustoni,* was studied by Barry (2). On methylation and hydrolysis of the polysaccharide 2,4,6-trimethylglucopyranose was obtained, showing that the glucose residues are

combined by 1,3-glycosidic linkages. The negative rotation of the laminarin suggests that the linkages are of the β-type. The belief that this polysaccharide exists in combination with sulfur as an ethereal sulfate could not be substantiated, inasmuch as extraction under mild conditions gave laminarin, which contained negligible amounts of combined sulfate. Hydrolysis of laminarin with oxalic acid or an enzyme from snail juice, produced a new disaccharide, for which the name "laminaribiose" was suggested (91). Its probable structure is glucose-3-β-glucoside. Formula IV represents the arrangement of the glucose residues in the laminarin.



IV. Arrangement of glucose units in laminarin, showing the 1,3-β-glucosidic linkages. [Cf. ref. 2.]

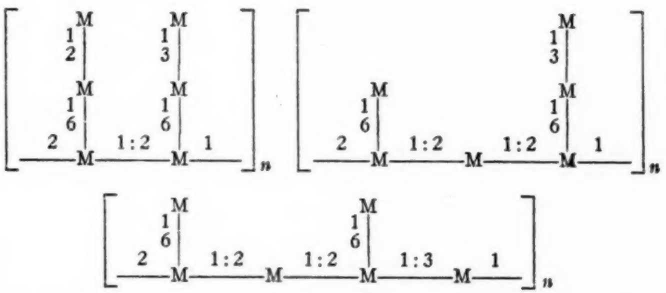
The extent of oxidation of laminarin with periodic acid was used by Barry (92, 93) as an "end-group assay" for this polysaccharide. The determination of this end group is based on the following: The periodic acid reacts with the group =C(OH)—C(OH)=, splitting the carbon bond and oxidizing each C·OH to CHO. In any polysaccharide in which the linkage is 1,4, as in starch or cellulose, the necessary pair of adjacent C·OH groups is provided by carbon atoms 2 and 3. If, however, carbon atom 3 takes part in the linkage of the units, there is no pair of adjacent C·OH groups available and the polysaccharide cannot be attacked by periodic acid. It was nevertheless found that a small but definite reduction of periodic acid took place when laminarin was used, apparently due to the oxidation of terminal nonaldehydic glucose units present in the molecule, inasmuch as these are the only units in the polysaccharide which could supply a =C(OH)—C(OH)= group for attack by the periodic acid. Subsequent oxidation with bromine yielded a polysaccharide whose end group contained two carboxyl groups. Estimation of the dicarboxylated end group indicated a chain length of 16 glucose units for laminarin. However, this chain length was not in agreement with that obtained by the Haworth-Hirst method of end-group assay, which indicated a chain length of about 74 glucose units.

An insoluble polyglucose having a similar β-type 1,3-glycosidic linkage occurs in yeast (*Saccharomyces cerevisiae*) (3). Hydrolysis of the methylated polysaccharide produced 2,4,6-trimethylglucose as the sole product. The laminarin and the yeast polyglucose are of special interest in that they are the only polysaccharides known in which the glucose residues are joined by 1,3-glycosidic linkages. Both of these polysaccharides are similar to cellulose or the amylose fraction from starch inasmuch as each is formed from one and the same kind of monosaccharide unit and consists of unbranched chains. They may, therefore, be considered "simple" polysaccharides, according to the classification suggested by Peat (6).

Yeast mannan.—The earlier work of Haworth, Hirst & Isherwood (94) on the structure of yeast mannan has been extended by Haworth, Heath & Peat (95). The last-named authors have shown that the mannan is homogeneous and consists entirely of *d*-mannopyranose residues. Hydrolysis of the methylated mannan results in a mixture of tetramethylmannopyranose, 3,4,6-trimethylmannose, 2,4,6-trimethylmannose, 2,3,4-trimethylmannose, and 3,4-dimethylmannose. If the three trimethylmannoses are grouped together as the "tri" fraction, it is found that the "tetra," "tri," and "di" fractions are present in equimolecular proportions. The greater part (90 per cent) of the "tri" fraction consists of equal parts of 3,4,6- and 2,4,6-trimethylmannose. A small amount of 2,3,4-trimethylmannose is also present. The latter trimethyl derivative constitutes not more than 10 per cent of the "tri" fraction or 3 per cent of the methylated mannan. The structure of yeast mannan, as interpreted from the relative proportion of these methylated derivatives, is represented by a central chain consisting of 1,2-glycosidically linked mannopyranose units with side chains attached to position 6. The small amount of 2,3,4-trimethylmannose obtained is considered to represent the nonreducing end group of the central chain. Optical rotation data indicate that the mannose residues are joined by α-glycosidic linkages. The diagrammatic Formula V represents some of the possible arrangements of the repeating unit. According to these investigators, the branched structures of six *d*-mannopyranose residues are probably repeated *n* times (the magnitude of *n* being 30 to 60) to form a terminated chain molecule of about 200 to 400 mannose residues.

Hemicelluloses.—Hemicelluloses usually refer to those water-insoluble complex polysaccharides in the cell walls, which may be extracted with dilute alkalis and hydrolyzed with dilute acids to their

constituent sugars and uronic acids. The hemicelluloses have no structural relation to cellulose. A complex polysaccharide described as a hemicellulose was extracted by Granichstädten & Percival (96) from Iceland moss, *Cetraria islandica,* with dilute sodium hydroxide. Acid hydrolysis of the polysaccharide produced a mixture of sugars containing 89 per cent glucose, 8 per cent galactose, 3 per cent mannose, and 5 per cent uronic acid, probably *d*-glucuronic acid. Methylation and fractionation, with molecular weight determination by the viscosity method, showed that the polysaccharide was not homogeneous. The hemicellulose is considered to be a mixture of polysaccharides made up chiefly of β-glucose units linked through positions 1,2, 1,3, 1,4, and 1,6—the first being a type of linkage thus far not reported for glucose units.



V. Yeast mannan. (Some of the possible representations of the repeating unit; M = *d*-mannopyranose.) [Cf. ref. 95.]

The hemicelluloses from sapwood of white pine, *Pinus strobus,* L. were studied by Anderson *et al.* (97). Hydrolysis of these hemicelluloses produced methoxyhexuronic acid, *d*-xylose, and *d*-mannose. The hemicelluloses isolated from the cottonwood, *Populus macdougali* (98) also consisted of methoxyuronic acid and *d*-xylose units, but, unlike those isolated from the white pine, contained no *d*-mannose. Small amounts of *d*-glucose are usually present along with the *d*-xylose among the hydrolysis products of the hemicelluloses. According to Anderson, it is not clear whether this *d*-glucose is derived from traces of starch or dextrin present as an impurity, or whether it is an integral part of the hemicellulose molecule. The methoxyhexuronic acid constituent of these wood hemicelluloses has not been identified with certainty,

but there are indications that it is probably methoxy-*d*-glucuronic acid. Since it is difficult to separate wood hemicelluloses into fractions of the same composition, they are probably mixtures of several chemical entities. The hemicelluloses from cottonwood (98) are considered to be mixtures of molecules consisting of chains varying in size from approximately seven to nine *d*-xylose units, each chain being combined with one molecule of monomethylhexuronic acid. These hemicellulose molecules are smaller than those obtained from hardwood (99). The white pine hemicelluloses (97) also are mixtures of molecules made up of a methylhexuronic acid unit combined with chains of five or six *d*-xylose units which in turn are combined with some *d*-mannose units. The positions involved in the linkage of the constituent monosaccharides to form the hemicellulose molecules are not determined. The hemicelluloses are not dissolved out of plant materials by water, but after they have been dissolved by alkali and reprecipitated by an acid they become soluble to some extent in water. According to Anderson, this suggests that the hemicelluloses are joined by an ester linkage between the carboxyl group of the uronic acid and an hydroxyl group of some cell-wall constituent, such as lignin or another hemicellulose molecule.

The hemicelluloses from sheep's fescue (*Festuca ovina*) and sweet vernal grass (*Anthoxanthum odoratum*) examined by Bennett (100) yielded on hydrolysis a uronic acid, *l*-arabinose, and *d*-xylose in the approximate ratios of 1 : 0.2 : 15.7 and 1 : 2.9 : 9.3, respectively. The cornstalk hemicelluloses studied by Weihe & Phillips (101) also consisted of uronic acid, *l*-arabinose, and *d*-xylose.

Mucilages.—Mucilages are similar to hemicelluloses in that they are also complex polysaccharides which on hydrolysis produce sugars and uronic acids. However, unlike the hemicelluloses, they are soluble in water. The mucilage, isolated by Anderson *et al.* (102) from indian wheat, *Plantago fastigiata,* contains *d*-xylose and an aldobionic acid, consisting of *d*-galacturonic acid and *l*-arabinose. The previously isolated mucilage from *Plantago psyllium* seed (103) is made up of the same constituents. No precise knowledge is available at present concerning the constitution of the aldobionic acid found in these mucilages. The mucilage extracted by Mullan & Percival (104) from the seeds of *Plantago lanceolata* likewise contains *d*-xylose and *d*-galacturonic acid, but differs from those isolated by Anderson *et al.* in that it also contains *d*-galactose and a methyl pentosan, and no *l*-arabinose. Methylation and hydrolysis of this mucilage yielded an

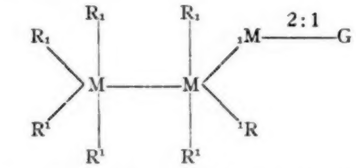
extraordinarily high proportion of trimethylxylopyranose (terminal groups), indicating a highly branched-chain molecular structure. The presence of 3,4-dimethylxylopyranose among the hydrolysis products of the methylated mucilage constitutes proof of the unusual 1,2-β-linkages of the xylose residues. That the glycosidic linkages are of the β-type is deduced from the fact that the polysaccharide, the acetate, and the methylated derivative have strong negative rotations. The galactopyranose units present in the molecule are linked through positions 1 and 3 as in agar (81). The mode of linkage of the uronic acid and the nature of the methyl pentose present is not determined.

The mucilage from the seeds of *Plantago arenaria* (105) consists of *d*-xylose, *l*-arabinose, *d*-galactose, and an aldobionic acid composed of *d*-galacturonic acid and *d*-xylose. Hydrolysis of the methylated polysaccharide produced trimethylxylopyranose (30 per cent), 2-methylxylose (23 per cent), tetramethylgalactopyranose (4 per cent), and a mixture (40 per cent) composed chiefly of 3,4-dimethylxylose and probably some methylated derivatives of arabinose. From these data it is concluded that the mucilage contains xylopyranose and galactopyranose end groups in the ratio of 9 to 1, and that the remaining xylose residues are glycosidically linked through 1,2-β-linkages, as in the case of the *Plantago lanceolata* mucilage.

Miscellaneous polysaccharides.—The carbohydrate fraction of ovomucoid which is present in egg albumin was studied by Stacey & Woolley (106). This carbohydrate is of interest because it is unique in that it is intermediate in size between an oligosaccharide and a polysaccharide. The carbohydrate fraction forming 20 per cent of the ovomucoid appears to be in combination with peptide residues. The fact that the latter are split off from the carbohydrate by saponification indicates that they are attached by ester linkages. Hydrolysis of the methylated carbohydrate fraction yields *N*-acetyl 3,4,6-trimethyl-*d*-glucopyranose (7 moles), *d*-mannopyranose (2 moles), 3,4,6-trimethyl-*d*-mannopyranose (1 mole), and 2,3,4,6-tetramethyl-*d*-galactopyranose (1 mole). Formula VI (p. 86) represents one of several possible methods of portraying the structure of this carbohydrate.

The carbohydrate is shown as a nonreducing hendecasaccharide having a central core of three mannose units to which seven *N*-acetylglucosamine units and one galactose unit are attached by glycosidic linkages. The presence of 3,4,6-trimethylmannopyranose among the cleavage products shows that one of the mannose residues in the molecule differs from the other two in that only one terminal group

is attached to it. This terminal unit is joined through a 1,2-glycosidic linkage and may be either galactose or *N*-acetylglucosamine as shown in Formula VI. The carbohydrate represented by this formula apparently cannot be regarded as a repeating unit but as one constituting the whole molecule. Because of its low molecular weight the ovomucoid carbohydrate is considered by these authors not as a polysaccharide, but rather as a hendecasaccharide. Indeed, the following facts support this view: the carbohydrate itself, its acetate, and its methylated derivative readily dialyze through parchment and collodion membranes; the methylated derivative shows low relative viscosities in chloroform solution, and both the acetate and the methylated derivative are easily soluble in water.



VI. Ovomucoid carbohydrate. (R = *N*-acetylglucosamine; M = *d*-mannopyranose; G = *d*-galactopyranose.) [Cf. ref. 106.]

Ford & Peat (107) isolated a water-soluble polysaccharide from wheat grain, which is associated with β-amylase. It was demonstrated that when the polysaccharide is separated from the enzyme the former loses its amylolytic activity, and that the functional part of the amylase is protein in nature. The polysaccharide is made up entirely of *l*-arabinose, *d*-xylose and *d*-galactose. Hydrolysis of the methylated polysaccharide yields trimethyl-*l*-arabofuranose (6 moles), 2,3-dimethylxylose (6 moles), 2-methylxylose (1 mole), xylose (1 mole) and 2,4-dimethylgalactose (1 mole). The polysaccharide is branched and bears some resemblance to vegetable gums (78), although, unlike the latter, it contains no acid constituents. It is essentially a xylo-araban in which the pentose units are mutually combined by 1,2-, 1,3-, and 1,4-glycosidic linkages with the last two predominating. The polysaccharide contains also a galactose residue attached at three points to other residues.

Further progress has been made by Reeves & Goebel (108) in elucidating the structure of the capsular polysaccharide of pneumococcus type III. While it was previously established that this poly-

saccharide consists of 4-β-glucuronosidoglucose or cellobiuronic acid units, the position of the hydroxyl group involved in the intramolecular linkage of similar aldobionic units in the polysaccharide remained an open question. The nature of these linkages has now been elucidated by the methylation method. Reeves & Goebel submitted the fully methylated polysaccharide to reduction with hydrogen at high temperature and pressure with barium-copper chromite as a catalyst. The carbomethoxyl groups of the methylated glucuronic ester residues were thus reduced to primary alcohol groups, and the methylated polysaccharide then consisted of a chain of methylated glucose residues only. Hydrolysis of this methylated polysaccharide yielded 2,3,6-trimethylglucose and a dimethylglucose, originally derived from the glucuronic acid constituent, which proved to be 2,4-dimethylglucose. The identification of this dimethyl derivative among the products of hydrolysis clearly shows that the cellobiuronic acid units in the polysaccharide are linked through positions 1 and 3. While only 1,3-glycosidic linkages are known to occur in some glucose polysaccharides (2,3), it is of interest to note that in the pneumococcus carbohydrate of type III the linkages alternate between positions 1,3 and 1,4. The configuration between the cellobiuronic acid units, as in the aldobionic acid itself, is assumed to be of the β-type. The structure of this polysaccharide may be diagrammatically represented by Formula VII.

$$\overset{4}{\text{——}}G\overset{1:3}{\text{————}}U\overset{1:4}{\text{————}}G\overset{1:3}{\text{————}}U\overset{1:4}{\text{————}}G\overset{1:3}{\text{————}}U\overset{1}{\text{——}}$$

VII. Polysaccharide of pneumococcus type III. (G = *d*-glucose; U = *d*-glucuronic acid.)

A water-soluble polysaccharide possessing immunological properties, which is produced by the fungus *Coccidioides immitis* (the causal agent of coccidioidomycosis in man and certain animals) was studied by Hassid, Baker & McCready (109). The polysaccharide was found to contain *d*-galacturonic acid, *d*-glucose, and some unidentified sugar in the approximate ratio of 1 : 6 : 3, respectively. A nitrogenous compound, apparently other than protein, is associated with this polysaccharide.

McIntire *et al.* (110) showed that a water-soluble polysaccharide producing only *d*-glucose on hydrolysis is formed by the crown-gall organism, *Phytomonas tumefaciens*. An upward shift in rotation during hydrolysis of this polysaccharide implied a predominance of β-

linkages. The rate and the smooth curve of hydrolysis indicated that the glucose residues have a pyranose structure. Its molecular weight calculated from sedimentation velocity and diffusion constants was 3600 ± 200.

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LITERATURE CITED

1. Haworth, W. N., Heath, R. L., and Peat, S., *J. Chem. Soc.*, 833–42 (1941)
2. Barry, V. C., *Sci. Proc. Roy. Dublin Soc.*, **22**, 59–67 (1939)
3. Hassid, W. Z., Joslyn, M. A., and McCready, R. M., *J. Am. Chem. Soc.*, **63**, 295–98 (1941)
4. Haworth, W. N., Raistrick, H., and Stacey, M., *Biochem. J.*, **31**, 640–44 (1937)
5. Hassid, W. Z., *J. Am. Chem. Soc.*, **61**, 1223–25 (1939)
6. Peat, S., *Ann. Repts. Progress Chem.*, **38**, 111–12 (Chem. Soc., London, 1941)
7. Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.*, 496–505 (1938); 452–60 (1939)
8. Bawn, C. E. H., Hirst, E. L., and Young, G. T., *Trans. Faraday Soc.*, **36**, 880–85 (1940)
9. Hirst, E. L., and Young, G. T., *J. Chem. Soc.*, 1471–82 (1939)
10. Hassid, W. Z., *Quart. Rev. Biol.*, **18**, 311–30 (1943)
11. Richardson, W. A., Higginbotham, R. S., and Farrow, F. D., *J. Textile Inst.*, **27T**, 131–57 (1936)
12. Freudenberg, K., Schaaf, E., Dumpert, G., and Ploetz, T., *Naturwissenschaften*, **27**, 850–53 (1939)
13. Hanes, C. S., *New Phytologist*, **36**, 189–239 (1937)
14. Kerr, R. W., *J. Am. Chem. Soc.*, **65**, 188–93 (1943)
15. French, D., and Rundle, R. E., *J. Am. Chem. Soc.*, **64**, 1651–53 (1942)
16. Rundle, R. E., and Edwards, F. C., *J. Am. Chem. Soc.*, **65**, 2200–3 (1943)
17. Rundle, R. E., and French, D., *J. Am. Chem. Soc.*, **65**, 1707–10 (1943)
18. Freudenberg, K., and Boppel, H., *Naturwissenschaften*, **28**, 264 (1940)
19. Freudenberg, K., and Boppel, H., *Ber. deut. chem. Ges.*, **73**, 609–20 (1940)
20. Barker, C. C., Hirst, E. L., and Young, G. T., *Nature*, **147**, 296 (1941)
21. Ahlborg, K., and Myrbäck, K., *Biochem. Z.*, **308**, 187–95 (1941)
22. Myrbäck, K., *Svensk. Kem. Tid.*, **52**, 126–33 (1940)
23. Meyer, K. H., and Bernfeld, P., *Helv. Chim. Acta*, **23**, 875–85 (1940)
24. Meyer, K. H., Wertheim, M., and Bernfeld, P., *Helv. Chim. Acta*, **23**, 865–75 (1940); **24**, 378–89 (1941)
25. Samec, M., and Waldschmidt-Leitz, E., *Z. physiol. Chem.*, **203**, 16–33 (1931)
26. Hess, K., and Krajnc, B., *Ber. deut. chem. Ges.*, **73**, 976–79 (1940)
27. Hess, K., and Steurer, E., *Ber. deut. chem. Ges.*, **73**, 1076–79 (1940)
28. Meyer, K. H., and Bernfeld, P., *Helv. Chim. Acta*, **23**, 845–53 (1940)
29. Meyer, K. H., Bernfeld, P., and Wolff, E., *Helv. Chim. Acta*, **23**, 854–64 (1940)
30. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, **65**, 1154–57 (1943)
31. Hassid, W. Z., and McCready, R. M., *J. Am. Chem. Soc.*, **65**, 1157–61 (1943)

32. Bates, F. L., French, D., and Rundle, R. E., *J. Am. Chem. Soc.*, **65**, 142–48 (1943)
33. Meyer, K. H., and Heinrich, P., *Helv. Chim. Acta*, **25**, 1038–46 (1942)
34. Meyer, K. H., and Heinrich, P., *Helv. Chim. Acta*, **25**, 1639–50 (1942)
35. Wolfrom, M. L., Smith, C. S., and Brown, A. L., *J. Am. Chem. Soc.*, **65**, 255–59 (1943)
36. Wolfrom, M. L., Myers, D. R., and Lassettre, E. N., *J. Am. Chem. Soc.*, **61**, 2172–75 (1939)
37. Wolfrom, M. L., and Myers, D. R., *J. Am. Chem. Soc.*, **63**, 1336–39 (1941)
38. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957–61 (1942)
39. Bear, R. S., *J. Am. Chem. Soc.*, **64**, 1388–92 (1942)
40. Kerr, R. W., and Severson, G. M., *J. Am. Chem. Soc.*, **65**, 193–98 (1943)
41. Wilson, E. J., Schoch, T. J., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1380–83 (1943)
42. Kerr, R. W., and Trubell, O. R., *Cereal Chem.*, **18**, 530–48 (1941)
43. Pacsu, E., and Mullen, J. W., *J. Am. Chem. Soc.*, **63**, 1168–69 (1941)
44. Meyer, K. H., and Fuld, M., *Helv. Chim. Acta*, **24**, 1408–9 (1941)
45. Wiegel, E., *Z. physik. Chem.*, **A188**, 137–59 (1941)
46. Foster, J. F., and Hixon, R. M., *J. Am. Chem. Soc.*, **65**, 618–22 (1943)
47. Levine, M., Foster, J. F., and Hixon, R. M., *J. Am. Chem. Soc.*, **64**, 2331–37 (1942)
48. Caldwell, C. G., and Hixon, R. M., *J. Am. Chem. Soc.*, **63**, 2876–80 (1941)
49. Hanes, C. S., *Proc. Roy. Soc. (London)*, **B129**, 174–208 (1940)
50. Hassid, W. Z., and McCready, R. M., *J. Am. Chem. Soc.*, **63**, 2171–73 (1941)
51. Haworth, W. N., Heath, R. L., and Peat, S., *J. Chem. Soc.*, 55–58 (1942)
52. Hassid, W. Z., Cori, G. T., and McCready, R. M., *J. Biol. Chem.*, **148**, 89–96 (1943)
53. Meyer, K. H., and Bernfeld, P., *Helv. Chim. Acta*, **24**, 389–93 (1941)
54. Rundle, R. E., and Baldwin, R. R., *J. Am. Chem. Soc.*, **65**, 554–58 (1943)
55. Rundle, R. E., and French, D., *J. Am. Chem. Soc.*, **65**, 558–61 (1943)
56. Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2954–56 (1942)
57. Lehrman, L., *J. Am. Chem. Soc.*, **64**, 2144–46 (1942)
58. Posternak, T., *Helv. Chim. Acta*, **18**, 1351–69 (1935)
59. Lampitt, L. H., Fuller, C. H. F., and Goldenberg, N., *J. Soc. Chem. Ind.*, **60**, 1–6, 25–29, 47–50, 69–72, 99–111, 137–41, 175–84, 231–41, 301–5 (1941)
60. Bear, R. S., and French, D., *J. Am. Chem. Soc.*, **63**, 2298–305 (1941)
61. Haworth, W. N., Hirst, E. L., and Smith, F., *J. Chem. Soc.*, 1914–22 (1939)
62. Baldwin, E., and Bell, D. J., *Biochem. J.*, **34**, 139–43 (1940)
63. Haworth, W. N., Hirst, E. L., and Isherwood, F. A., *J. Chem. Soc.*, 577–81 (1937)
64. Morris, D. L., and Morris, C. T., *J. Biol. Chem.*, **130**, 535–44 (1939)
65. Hassid, W. Z., and McCready, R. M., *J. Am. Chem. Soc.*, **63**, 1632–35 (1941)
66. Meyer, K. H., and Fuld, M., *Helv. Chim. Acta*, **24**, 375–78 (1941)

67. Meyer, K. H., *Advances in Enzymology,* **III**, 109–35 (Interscience Publishers, Inc., New York, 1943)
68. Carter, S. R., and Record, B. R., *J. Chem. Soc.,* 664–75 (1939)
69. Hess, K., and Neumann, F., *Ber.,* **70**, 728–33 (1937)
70. Haworth, W. N., Hirst, E. L., Owen, L. N., Peat, S., and Averill, F. J., *J. Chem. Soc.,* 1885–99 (1939)
71. Haworth, W. N., Montonna, R. E., and Peat, S., *J. Chem. Soc.,* 1899–901 (1939)
72. Haworth, W. N., *Chemistry & Industry,* **58**, 917–25 (1939)
73. Carrington, H. C., Haworth, W. N., Hirst, E. L., and Stacey, M., *J. Chem. Soc.,* 1901–4 (1939)
74. Haworth, W. N., Peat, S., and Wilson, W. J., *J. Chem. Soc.,* 1904–8 (1939)
75. Godman, G. L., Haworth, W. N., and Peat, S., *J. Chem. Soc.,* 1908–14 (1939)
75a. Gardner, T. S., and Purves, C. B., *J. Am. Chem. Soc.,* **64**, 1539-42 (1942); Mahoney, J. F., and Purves, C. B., *J. Am. Chem. Soc.,* **64**, 9–15 (1942)
76. Peterson, F. C., Barry, A. J., Unkauf, H., and Wise, L. E., *J. Am. Chem. Soc.,* **62**, 2361–65 (1940)
77. Hirst, E. L., Jones, J. K. N., and Campbell, W. G., *Nature,* **147**, 25–26 (1941)
78. Hirst, E. L., *J. Chem. Soc.,* 70–78 (1942)
79. Mosimann, H., and Svedberg, T., *Kolloid-Z.,* **100**, 99–105 (1942)
80. White, E. V., *J. Am. Chem. Soc.,* **63**, 2871–75 (1941); **64**, 302–6 (1942); **64**, 1507–11 (1942); **64**, 2838–42 (1942)
81. Percival, E. G. V., and Somerville, J. C., *J. Chem. Soc.,* 1615–19 (1937)
82. Pirie, N. W., *Biochem. J.,* **30**, 369–73 (1936)
83. Hands, S., and Peat, S., *Chemistry & Industry,* **57**, 937–38 (1938); *Nature,* **142**, 797 (1938); Percival, E. G. V., Somerville, J. C., and Forbes, I. A., *Nature,* **142**, 797–98 (1938); Forbes, I. A., and Percival, E. G. V., *J. Chem. Soc.,* 1844–49 (1939); Cottrell, T. L., and Percival, E. G. V., *J. Chem. Soc.,* 749–50 (1942)
84. Araki, T., *Chem. Abstracts,* **37**, 91 (1943)
85. Duff, R. B., and Percival, E. G. V., *J. Chem. Soc.,* 830–33 (1941)
86. Jones, W. G. M., and Peat, S., *J. Chem. Soc.,* 225–31 (1942)
87. Percival, E. G. V., and Thomson, T. G. H., *J. Chem. Soc.,* 750–55 (1943)
88. Buchanan, J., Percival, E. E., and Percival, E. G. V., *J. Chem. Soc.,* 51–54 (1943)
89. Baldwin, E., and Bell, D. J., *J. Chem. Soc.,* 1461–65 (1938)
90. Bell, D. J., and Baldwin, E., *Nature,* **146**, 559–60 (1940); *J. Chem. Soc.,* 125–32 (1941)
91. Barry, V. C., *Sci. Proc. Roy. Dublin Soc.,* **22**, 423–29 (1941)
92. Barry, V. C., Dillon, T., and McGettrick, W., *J. Chem. Soc.,* 183–85 (1942)
93. Barry, V. C., *J. Chem. Soc.,* 578–81 (1942)

94. Haworth, W. N., Hirst, E. L., and Isherwood, F. A., *J. Chem. Soc.*, 784–91 (1937)
95. Haworth, W. N., Heath, R. L., and Peat, S., *J. Chem. Soc.*, 833–42 (1941)
96. Granichstädten, H., and Percival, E. G. V., *J. Chem. Soc.*, 54–58 (1943)
97. Anderson, E., Kesselman, J., and Bennett, E. C., *J. Biol. Chem.*, **140**, 563–68 (1941)
98. Anderson, E., Kaster, R. B., and Seeley, M. G., *J. Biol. Chem.*, **144**, 767–72 (1942)
99. Anderson, E., Seeley, M., Stewart, W. T., Redd, J. C., and Westerbeke, D., *J. Biol. Chem.*, **135**, 189–98 (1940)
100. Bennett, E., *J. Biol. Chem.*, **146**, 407–9 (1942)
101. Weihe, H. D., and Phillips, M., *J. Agr. Research*, **64**, 401–6 (1942)
102. Anderson, E., Gillette, L. A., and Seeley, M. G., *J. Biol. Chem.*, **140**, 569–74 (1941)
103. Anderson, E., and Fireman, M., *J. Biol. Chem.*, **109**, 437–42 (1935)
104. Mullan, J., and Percival, E. G. V., *J. Chem. Soc.*, 1501–6 (1940)
105. Nelson, W. A. G., and Percival, E. G. V., *J. Chem. Soc.*, 58–61 (1942)
106. Stacey, M., and Woolley, J. M., *J. Chem. Soc.*, 184–91 (1940); 550–55 (1942)
107. Ford, L. H., and Peat, S., *J. Chem. Soc.*, 856–64 (1941)
108. Reeves, R. E., and Goebel, W. F., *J. Biol. Chem.*, **139**, 511–19 (1941)
109. Hassid, W. Z., Baker, E. E., and McCready, R. M., *J. Biol. Chem.*, **149**, 303–11 (1943)
110. McIntire, F. C., Peterson, W. H., and Riker, A. J., *J. Biol. Chem.*. **143**, 491–96 (1942)

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THE CHEMISTRY OF THE LIPIDS

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The lipids, as recently defined by Bloor (1), include those groups of "naturally occurring substances consisting of the higher fatty acids, their naturally occurring compounds, and substances found naturally in chemical association with them." In the last two volumes of the *Annual Review of Biochemistry,* the chemistry of the acyclic constituents of the fats and oils (up to October, 1941) was reviewed by Hilditch (2), and the chemistry of the lipins by Thannhauser & Schmidt (3). The present article continues to report the progress of lipid research, excepting, however, certain classes of compounds usually regarded as lipids, namely, the sterols and the fat-soluble vitamins and hormones, which are discussed elsewhere. Further, the limitations of space prohibit including a large amount of work on another group of lipids, the anti-oxidants.

The most important publication of the past year related to this subject is a new book, *The Biochemistry of the Fatty Acids and Their Compounds, the Lipids,* by W. R. Bloor (1). This book together with Hilditch's *The Chemical Constitution of the Natural Fats,* published in 1940 (4), and the Second Edition of Jamieson's *Vegetable Fats and Oils* (5) affords an excellent coverage of this subject. The subjects of the six chapters of Bloor's book, which describe the scope of its contents, are as follows: I. Chemistry, Descriptive and Analytical; II. Digestion and Absorption; III. Lipids of the Blood; IV. Lipids in Tissue; V. Lipid Metabolism; VI. The Lipids of Secretions and Excretions. Extensive bibliographies are given at the end of each chapter and include literature up to about 1940. This book, coming as it does from an outstanding investigator in the field, is most welcome. One of its important services, however, will be the challenge to further work, which will be the natural result of surveying the incomplete and usually conflicting state of our knowledge in most of the phases of lipid chemistry.

The reader is also referred to the annual reviews on the chemistry of the fats and fatty acids, which appear usually in March and April of each year in *Oil and Soap,* the official journal of the American Oil Chemist's Society (6).

Methods of Examining the Fatty Acids and Glycerides

No important new methods of analysis of the fats and fatty acids have appeared. Interest has been directed to the modification and improvement of previously reported procedures, with special emphasis on the separation of fatty compounds by chromatographic adsorption. There have also been important developments in the application of spectroscopy to the analysis of lipid mixtures.

An interesting double indicator method for the determination of the saponification number has been described by Rieman (7). The fat is saponified by an excess of alcoholic potash, phenolphthalein is added, and the solution is titrated with 0.5 *N* hydrochloric acid to the disappearance of indicator color. Bromophenol blue and benzol are then added and the solution is titrated with the acid to a green color which persists after shaking, this second volume of acid being used in the calculation. By this procedure the use of blanks is avoided. Good agreement is claimed with the standard method.

Continued interest has been shown in procedures for the determination of iodine numbers of fats and oils containing conjugated double bonds. For example, although Hanus solution in large excess measures the total unsaturation of dehydrated castor oil which contains conjugated octadecadienoic acids, it is unsuitable for other conjugated oils and fatty acids. Von Mikusch & Frazier (8) showed that higher concentrations of iodine bromide, as in the Woburn solution, which is 0.32 *N*, give results representing total saturation in tung oil and in such fatty acids as 9,11-linoleic acid. They later proposed the use of total and partial iodine values as a method for indirect determination of the diene value (9). The total iodine value is determined by the Woburn solution and the partial iodine value by the Wijs solution. The following relation exists: total iodine value minus partial iodine value equals diene value. Norris & Buswell (10) described rapid methods (3 to 5 mins.) for determination of the iodine number in which mercuric acetate is added to the reagent, either Wijs or Hanus. With the rapid Hanus method, for example, excellent results were obtained on a number of unconjugated fats and oils, but the method was unsatisfactory for castor oil. A modified Ellis-Jones maleic anhydride procedure has been described by McKinney, Halbrook & Rose (11). Kaufmann *et al.* (12) have shown that tetranitromethane in chloroform or carbon tetrachloride solution reacts with unsaturated fats and fatty acids to give a red color, which de-

velops in proportion to the iodine number. This reagent causes elaidinization in one to two days.

Investigators in this field are employing increasingly the thiocyanogen reaction in the analysis of fats and fatty acids. Kaufmann & Grosse-Oetringhaus (13) found that thiocyanogen is stable in benzol, toluol, pentachloroethane, and tetrachloroethylene, while it is unstable in methyl cyclohexane, nitrobenzol, and formic acid. Thiocyanogen values were most reliable in glacial acetic acid. Several groups of investigators have recently taken up the question of the true thiocyanogen values of oleic, linoleic, and linolenic acids (14 to 17) with the general conclusion that the values are empirical. Riemenschneider, Swift & Sando (18) determined the thiocyanogen numbers of the methyl esters of these acids in 0.1 and 0.2 *N* reagent. As a result of their work they suggest the following values with 0.2 *N* reagent: oleic, 89.4; linoleic, 96.8; and linolenic, 167.5. The latest contribution to this problem is by Painter & Nesbitt (19) who give an excellent discussion of previous work and propose 89.9, 98.1, and 168.0 as the thiocyanogen values of the three acids, respectively. From simultaneous equations based on these values, they found 39.4 to 59.1 per cent of linolenic acid in various linseed oils which they examined.

Mitchell & Kraybill (20) found that bleaching of vegetable oils with fuller's earth caused absorption bands to develop at 2680 Å, 3000 Å and 3160 Å. Only the first of these bands developed in oils containing acids with no greater unsaturation than linoleic acid, but when linolenic acid was present, the latter two bands developed, which fact indicates tetraene unsaturation. The total amount of absorptive material amounted to only 0.1 to 0.2 per cent, and was believed to be due to preliminary oxidation followed by dehydration. Mitchell, Kraybill & Zscheile (21) have developed a complete spectral analysis for fats. After isomerization by heating with alkali in glycol at 180°, the amounts of linoleic and linolenic acids are estimated by means of absorption spectra. Oleic acid is determined from the difference between the iodine number of the original mixture and the calculated iodine number of the linoleic and linolenic acids in the mixture; finally, the saturated acids are calculated by subtracting the total content of unsaturated acids from 100 per cent. Results of analyses of synthetic mixtures of known composition were satisfactory. A comprehensive recent publication on infrared spectroscopy by Barnes, Liddel & Williams (22) includes absorption curves of a number of fatty acids, esters, fats, and oils.

Chromatographic adsorption has been applied to the separation of fatty acids and glycerides. Kaufmann (23) passed a 1 : 1 mixture of myristic and stearic acids in ten parts of benzol over aluminum oxide; myristic acid alone appeared in the filtrate. Cassidy (24) described the separation of mixtures of lauric, myristic, palmitic, and stearic acids in petroleum ether by passing them over carbon. A difference of two carbon atoms in the components of the mixture required about ten parts of carbon per part of mixture. Graff & Skau (25) separated mixtures of stearic and oleic acids and of stearic and myristic acids by passing them over a heavy magnesium oxide column impregnated with a suitable indicator. Colored chromatograms were produced with phenol red as the dye. An interesting instance of adsorption of palmitic acid dissolved in methyl alcohol by corn and potato starches is reported by Lehrman (26). The separation of trimyristin and tristearin can be accomplished by passing chloroform solutions of these fats over alumina or silica gel (27). Claesson (28) separated the glycerides of the higher saturated acids and oleic acid by passing ether solutions over active carbon, but the process was ineffective for free fatty acids. One of the most detailed chromatographic investigations of a natural oil has been reported by Walker & Mills (29), who by repeatedly passing linseed oil in hexane over alumina columns, obtained six fractions of iodine number 117.7 to 246.5. The fraction of highest iodine number was calculated to contain 47.8 per cent trilinolenin and 52.2 per cent linoleo-dilinolenin, and thus afforded proof of the presence of the above triglycerides in this oil.

Swift, Rose & Jamieson (30) prepared methyl linoleate of 97.7 per cent purity by passing the methyl esters of cottonseed oil in petroleum ether over alumina. The operation was carried out in two steps; the first passage over the adsorbent resulted in a product of iodine number 159.5, and the second in the nearly pure ester. This finding is especially important in that it constitutes a second type of purely physical method for the preparation of this dienoic acid, the other being the method of low temperature crystallization. Fitelson (31) applied chromatographic adsorption to the determination of the squalene content of twenty-three fats. By passing the unsaponifiable fraction from these fats over a column of alumina, the squalene was concentrated in the unadsorbed filtrate and was isolated as the hexahydrochloride. The squalene contents ranged from 0 for cocoa butter to 708 mg. per 100 gm. for olive oil. Moderately high values were shown for corn, peanut, and rice bran oils.

Schuette & Vogel (32) reviewed the role of solidification points of binary mixtures of fatty acids from C_{10} to C_{24} in the identification and analysis of mixtures. Application of this method to the margaric acid (C_{17}) of alfalfa seed oil showed it to be a mixture of palmitic and stearic acids. Schuette, Christenson & Vogel (33) synthesized the even carbon fatty acids from C_{24} to C_{30} and determined the solidification point curves of the binary mixtures, C_{24}–C_{26}, C_{26}–C_{28}, and C_{28}–C_{30}. Solidification point (34) and refractive index (35) nomographs have been published by Davis. Hoerr, Pool, & Ralston (36) observed the effect of water on the solidification points of the saturated fatty acids. For example, stearic and myristic acids containing 1 per cent water showed depression of the solidification points of 0.43° and 0.8° respectively. Solubilities of water in a number of the saturated fatty acids were determined. At 90° lauric acid dissolved 2.85 per cent of water and at 92° stearic acid dissolved 1.02 per cent.

A second bibliography on molecular distillation, including 190 references, has been prepared by Todd (37). An improved distillation apparatus for the efficient separation of small quantities of fatty acid esters has been described by Klenk & Schuwirth (38). Schuwirth (39) devised a still, suitable for use at pressures of 10^{-3} and 10^{-4} mm., with which he was able to demonstrate the presence of a hexadecenoic acid in the unsaturated fatty acids of the glycerol phosphatides of human brain.

The hydroxamic acid derivatives have been used by Inoue *et al.* (40) to separate the saturated and unsaturated acids of soybean oil. The hydroxamic acids are extracted with alcohol and other solvents. The acids of the insoluble and filtrate fractions gave iodine numbers of 12.9 and 149.5, respectively. By means of these derivatives it was claimed that pure linoleic and linolenic acids were prepared (41).

Low temperature crystallization as a method for the separation of saturated and unsaturated acids, proposed by Brown & Stoner (42) in 1937, and adapted to the determination of the saturated acids of soybean oil by Earle & Milner (43), has been further employed for the determination of the saturated acids of a number of seed oils by Anthony, Quackenbush & Steenbock (44). The acids of the oil in question were separated into three fractions by crystallization from acetone at —40°: a saturated fraction of average iodine number 3.0, an intermediate fraction of iodine number 75, and a filtrate fraction. From the data, the content of saturated acids was calculated. Analyses of fifteen oils were reported, including values for the unsaturated acids

by the iodine-thiocyanogen equations. Cramer & Brown (45) have further developed the low temperature crystallization method in a study of the component fatty acids of human depot fat. The methyl esters of this fat were fractionated through an efficient column. The resultant main fractions were then separated into their component esters by crystallization procedures. A number of pure esters were thus prepared from this fat. Additional examples of the applications of the low temperature crystallization and ester fractionation procedures are to be found later in this review.

Stetten & Grail (46) developed a method for microtitration of fatty acids, suitable for determining 8 to 20 mg. Apparatus was described for carrying out this titration, and, also, for microextraction of the original lipids. Estimation of plasma fatty acids is feasible with the method.

The Fatty Acids

The conversion of oleic acid to palmitic by heating with potassium hydroxide has been known for many years. Farmer (47) has shown that this reaction under strict control can be made to yield principally a mixture of octadecenoic acids with a shift of position of the double bond always toward the carboxyl group. Two unusual octadecenoic acids, with the double bond in the 16:17, and 17:18 positions, have been synthesized by Kapp & Knoll (48). The melting points of these acids are 62.8–63.5°, and 55.0–55.5°, respectively. By selective hydrogenation of linoleic and linolenic acids, Bömer & Stather (49) obtained 10,11-octadecenoic acid which was separated from the resultant mixture of acids by fractional crystallization of the lead and magnesium salts. The acid melted at 35°.

Several important studies of the oxidation products of the monoethenoic acids have been reported. Kass & Radlove (50) described the preparation of ricinoleic and ricinelaidic acids. The properties of the trihydroxy acids prepared by oxidation of these unsaturated acids afforded further evidence for the cis structure for ricinoleic acid and the trans structure for ricinelaidic acid. King (51) showed that the naturally occurring dihydroxy stearic acid from castor oil is a 9,10-dihydroxy acid, melting at 141°, and, further, that it is one of the components of the racemic mixture of dihydroxy acids, melting at 132°, usually obtained by oxidation of oleic acid. However, the optical activity of the stereoisomer was too feeble for accurate measurement. King (52), as a result of oxidizing oleic and elaidic acids with

hydrogen peroxide in acetic acid, concluded that fission of the oxygen ring, whether by hydrolysis or by union with acid, is accompanied by an inversion of configuration. Hilditch & Plimmer (53) investigated the oxidation products of the 9,10-hydroxyketostearic acids and found that increasing the excess of alkali present favored the formation of the dihydroxystearic acid melting at 132°. Atherton & Hilditch (54), in studying the stereochemical relationships of the oxido- and dihydroxystearic acids, concluded that no inversion takes place during the conversions of oleic and elaidic acids into the dihydroxy acids with perbenzoic, peracetic, or Caro's acids. A detailed study of the oxidation products of erucic and brassidic acids was made by Dorée & Pepper (55). They prepared pure erucic acid from rapeseed oil, and, by elaidinization, the trans isomer, brassidic acid. By oxidation of erucic acid with hydrogen peroxide in acetic acid at 100° a mixture of dihydroxybehenic acid, melting at 100°, and of oxidobrassidic acid, (70.5°) was obtained. However, by use of hydrogen peroxide in acetic acid plus osmium tetroxide or with alkaline permanganate, the dihydroxybehenic acid, melting at 132°, was the principal product. The oxidation products from brassidic acid were exactly the reverse with these reagents.

Henderson & Young (56) measured the induction periods and oxygen absorption rates of a number of preparations of oleic acid. The induction period at 80° was about five hours and was little affected by varying the concentration of oxygen. Peroxide formation was the initial reaction. An empirical rate law was set up and verified for the early stages of the reaction.

The ease of hydrogenation of the octadecenoic acids is affected by the position of the double bond. The least reactive is the 2,3-octadecenoic acid, while the 3,4-, 6,7- and 9,10- acids are increasingly easily hydrogenated (57).

Frankel & Brown (58) prepared α- and β-linoleic acids by reduction of the corresponding bromides with zinc in several solvents. Ether was found to be the best medium for the reduction reaction with resultant high yields and no ester formation, as is the case when an alcohol is used. The properties of the α and β acids were compared. It was concluded that the α-acid was composed mainly of cis,cis 9,12-octadecadienoic acid (linoleic acid), but also contained varying amounts of isomeric octadecadienoic acids, depending on the debromination conditions. The β-linoleic acid preparations were shown to contain 15 to 53 per cent linoleic acid, 32 to 70 per cent of isomeric octadeca-

dienoic acids, and 2 to 6 per cent of conjugated material. Frankel, Stoneburner & Brown (59), employing the procedure described in 1941 (60), have isolated pure linoleic acid by low temperature crystallization of the fatty acids of sesame, cottonseed, grapeseed, and poppyseed oils. The preparations were adjudged to be pure on the basis of iodine number and melting point, both of which agreed with the values obtained for the specimen of highly purified linoleic acid prepared by Matthews *et al.* (61). The tetrabromide numbers of these specimens, however, were slightly lower than the value 102.9, which finding indicates the possible presence of very small amounts of isomeric acids. This is the first time that linoleic acids have been prepared from these oils by physical methods alone. The fatty acids of olive oil yielded a linoleic acid preparation of low tetrabromide number from which it was concluded that the linoleic acid of this oil is a mixture of octadecadienoic acids.

By ozonolysis of ethyl linoleate, Arcus & Smedley-Maclean (62) obtained products which further confirm the 9,12 structure of linoleic acid. Von Mikusch (63) has isolated a new isomer of linoleic acid from the reaction products of dehydrated castor oil and aqueous sodium hydroxide. The 10,12-octadecadienoic acid, thus formed, melted at 57° and apparently arose from the reaction of the alkali on the linoleic acid in the original oil.

Detailed directions for the preparation of linoleic acid from sunflower seed oil (64), and of linolenic acid from linseed oil (65) have been given by McCutcheon. These acids were made by reduction of the corresponding bromides in absolute alcohol to give the ethyl esters, from which the acids were prepared by the usual procedures.

A further confirmation of the structure of arachidonic acid, originally proposed by Dolby, Nunn & Smedley-Maclean (66) as 5,8,11,14-eicosatetrenoic acid, and verified by Mowry *et al.* (67), has been reported by Arcus & Smedley-Maclean (62) who submitted a specimen of debromination arachidonic acid to disruptive oxidation. Their results entirely support the proposed structure. It is clear from these several researches that this acid, whether prepared by debromination of the octabromide or by purely physical methods (68) is composed for the most part of the single isomer, as noted above, but it is likely that small amounts of contaminating isomers occur along with the predominating component which we call arachidonic acid.

According to Mowry, Brode & Brown (67), hydrogenation of methyl arachidonate proceeds in two stages, a diethylenic product

being obtained as an intermediate; this consists of 5,14-eicosadienoate (80 per cent) and 8,14-eicosadienoate (20 per cent).

Burr *et al.* (69) have continued their work on the effect of heat on unsaturated esters. A temperature of 150° for 24 hours produced no demonstrable effect on the unsaturated esters of cod liver oil, but at 200° a detectable increase in absorption at 2350 and 2700 Å occurred in six hours after which the absorption decreased; this indicates a disappearance of absorbable material. Linseed oil esters and methyl linolenate were fairly resistant to rearrangement during fractional distillation; only two double bond conjugations appeared in linseed oil esters and three double bond conjugations in cod liver oil esters. These results are borne out further by the observation of Millican in this laboratory (70) that linseed oil esters can be fractionated successfully through an electrically heated column packed with glass-helices; no apparent alteration results, as evidenced by constancy of iodine number and the presence of little or no residue in the still pot. On the other hand, Ault *et al.* (71) have described the polymerization of esters of several of the octadecatrienoic acids at 275°. The two conjugated esters, the a- and b-eleostearates, polymerized faster than the two non-conjugated esters studied. Evidence was presented in support of the diene mechanism of polymerization, including proposed structures of a bicyclic trimer and a tricyclic bimer. Bradley & Richardson (72) described the formation of highly conjugated systems by heating drying oils and their fatty acids at 225° with 37 to 50 per cent alkali.

Tutiya (73) has assigned the formula, 9,11,13,15-octadecatetrenoic acid to the parinaric acid of the seed oil of Hosenka. Possible structures for the hexadecatetrenoic and octadecatrienoic acids of sardine oil (74) were also suggested. The bromide of the latter acid melted at 215°. Confirmation of the presence of an isomer of linolenic acid in a fish oil will be awaited with interest.

The preparation of several fatty acid monoesters of ascorbic acid and of *d*-isoascorbic acid was carried out by Swern, Stirton, Turer & Wells (75). Only the primary hydroxyl groups of the ascorbic acids were esterified in these compounds.

Chemistry of the Glycerides. The Fats and Oils

The reviews by Piskur (6), previously mentioned, include tables of general analytical data on the fats and oils and, in many instances, details of fatty acid composition.

Important information with a minimum of analytical effort is being obtained on many fats and oils by application of the iodine-thiocyanogen simultaneous equations, applied to the glycerides, the mixed fatty acids, or to the mixed methyl esters. With the recent modifications of the original Kaufmann equations (see preceding section), it is possible to obtain satisfactory results on the content of saturated oleic, linoleic, and linolenic acids, or, to be more precise, the content of total saturated, monoethenoic, diethenoic, and triethenoic acids. No attempt has been made to use the method for more highly unsaturated acids. It is to be noted further that when acids of one, two, and more than two, double bonds are present, it is necessary to determine the amounts of saturated acids independently.

In connection with the analysis of biological materials for total fat, increasing interest is being shown in the problem of attaining complete extraction. In studies of the determination of total fat in commercial feeds, Randle (76) and Schall & Thornton (77) have shown that preliminary hydrolysis by hydrochloric acid followed by petroleum ether extraction gives results considerably higher (20 per cent) than extraction by ether alone. Harrison (78) determined the efficiency of extraction of fish meal by a considerable series of fat solvents with the conclusion that "none of the solvents, or mixtures of solvents, or procedures investigated gives values that can be considered entirely accurate measures of fat content." Grossfeld (79) has suggested 60 to 70° benzine as a suitable extractant. Schloemer & Rauch (80) determined the fat in milk by acid hydrolysis in the presence of carbon tetrachloride or trichloroethylene. Other phases of this problem, especially in relation to tissue lipo-proteins, are discussed later.

While it is not within the scope of this review to cover the many published papers describing the advances in food technology included in the general subject of hydrogenation of fats, four of these papers will be mentioned. Vahlteich (81) has discussed the advances in hydrogenation which are being applied to the development of quality in margarine, which of necessity is now coming into increased use in the American economy. Handschumaker, Thompson & McIntyre (82) have partially separated the glycerides of various hydrogenated fats by crystallization from acetone and ether, and have given some data on the constituent linoleins. By the use of iodine-thiocyanogen equations, Andrews & Richardson (83) analyzed a large number of shortenings and other edible fats for their content of linoleic acid. Because

of the importance of their findings in nutrition, these are summarized in part in Table I.

TABLE I

PERCENTAGE OF LINOLEIC ACID IN EDIBLE FATS*

Samples	Lard	Butter	Margarine	Hydrogenated		Mixed Animal and Vegetable Fats
				100%	<100%	
Number of samples.	27	41	57	60	31	11
High	13.7	4.8	23.4	22.4	38.2	26.2
Low	6.5	1.4	1.3	2.9	4.7	10.4
Median	11.8	3.3	9.9	12.8	21.5	23.2
Average	11.7	3.3	10.9	12.6	22.0	20.6

* From Andrews & Richardson (83).

It should be noted that the linoleic acid values in Table I are total octadecadienoic acids. Butterfat, for example, has been repeatedly shown not to contain appreciable amounts of ordinary linoleic acid. Apparatus for the low temperature crystallization of glycerides was described by Bailey and co-workers (84). They obtained a product resembling olive oil by partial hydrogenation of peanut oil until its oleic/linoleic acid ratio was about 92 to 98, followed by partial crystallization at low temperatures.

The various physical and chemical methods for investigation of the component glycerides in natural fats have been reviewed by Kaufmann & Kirsch (85). In addition to fractional crystallization, the chemical methods which they noted are reesterification, elaidinization, oxidation, hydrogenation, and bromination, and also the newer method of adsorption.

Whale oil was fractionally crystallized by Hilditch & Maddison (86) by dissolving in acetone and cutting into four fractions at —10°, —20°, and —30° and filtrate. They concluded that over half the glycerides in the oil contained no acid higher than C_{18} and that one-third of the glycerides contained a C_{20} or a C_{22} highly unsaturated acid. Baldwin & Parks (87) employed low temperature crystallization in the separation of the glycerides of menhaden oil into simplified fractions. One of their —60° filtrate fractions had an iodine number of 268. However, as is the case with most natural fats, the even distribution of the fatty acids over the glyceride molecules prevents a wider range of separation. This is further borne out by the experience of

Bull & Wheeler (88), who were able to obtain 30 to 60 per cent yields of comparatively unsaturated glycerides of iodine number 145 to 155 by crystallization of soybean oil from acetone at temperatures as low as —70°. On the other hand, by crystallization of the fatty acids themselves, they obtained fractions of iodine number up to 190. Solvent extraction of linseed oil, corn oil, cottonseed oil, and other oils with methanol was employed successfully by Kleinsmith & Kraybill (89) in the separation of unsaturated fractions of superior drying properties.

Hilditch & Meara (90) have defined "the rule of even distribution" as follows:

1. If any individual acid (e.g., oleic) forms one-third or more of the total molecules of fatty acid present in the fat, that acid will occur once (or more than once) in nearly all the triglyceride molecules. 2. Any (minor component) acid which does not form more than about ten per cent of the total molecules of fatty acids will contribute only one acyl group to such triglyceride molecules as may contain it.

They discussed in detail the computation of the mixed glycerides in natural fats from the fatty acid composition. The glyceride structure of butter fat as influenced by dietary fat was studied by Hilditch & Jasperson (91). For example, when peanut oil or peanut oil hydrogenated to iodine number 45 was fed, the proportions of oleo-glycerides were increased and the C_4 to C_{10} glycerides decreased. Other contributions to our knowledge of glyceride structure were made by Hilditch & Zaky (92) working with shorea robusta kernel fat and by Hilditch, Sime & Maddison (93) on the fat of kangaroo and Ceylon bear. In the latter paper several wild animal fats and two bird fats were examined for component fatty acids by ester fractionation. The kangaroo and bear fats were separated into fractions by crystallization before application of ester fractionation.

Lovern (94) has reviewed the composition of the depot fats of wild animals. In a study of a considerable series of goat milk fats, Zeisset & Grossfeld (95) found no significant differences from different breeds.

Detailed fatty acid analyses, usually by the ester fractionation method, have been reported as follows: bone marrow of the reindeer, including results both by ester fractionation and by iodine-thiocyanogen methods (96); avocado pulp oil (97); arrow wood (98); watermelon seed (99), 60.1 per cent linoleic acid; ragweed seed (100), 69.8 per cent linoleic acid; Mexican prickly poppy seed (101), 58.6

per cent linoleic acid; safflower oil (102), 61.0 per cent linoleic acid; guanabana seed oil (103); and tropical almond (104). Analyses of five specimens of human depot fat, previously mentioned (45), gave the following range of results: lauric 0 to 0.9 per cent; myristic 2.6 to 5.9; tetradecenoic, 0.2 to 0.6; palmitic, 24.0 to 25.7; hexadecenoic, 5.0 to 7.6; stearic, 5.2 to 8.4; octadecenoic, 44.8 to 46.9; octadecadienoic, 8.2 to 11.0; arachidonic, 0.3 to 1.0; other C_{20} acids, 0.3 to 2.2. Several of the fatty acids in human fat were prepared in a pure state by crystallization procedures for the first time. Evidence was presented for the occurrence of isomeric octadecenoic and octadecadienoic acids, in addition to ordinary oleic and linoleic acids.

In several investigations, important information on the component fatty acids has been obtained mainly by application of the iodine-thiocyanogen equations. These include the following: black bear (105); beef and pig bone marrow fat (106); lard and beef, veal and mutton tallows including contents of vaccenic (octadecenoic) acid amounting to 0.4 to 3.4 per cent (107); chicken and turkey fats (108), 20 per cent linoleic; also the following high linoleic acid oils: beechnut (109), 41.9 per cent linoleic; road thistle, *Onopordon acanthum* L (110), 64 per cent linoleic; rye embryo oil (111), 63.3 per cent linoleic; Bulgarian tobacco seed (112), 65.6 per cent linoleic; apricot kernel oil (113); and Dutch night violet, *Hesperis matronalia* L (114). Eel fat is of especial interest because of its content of linolenic acid, 17.0 per cent (115).

Painter & Nesbitt (116) have examined a large number of specimens of linseed oil, the contents of linolenic acid ranging from 30.4 to 65.2 per cent. In general, most of their values are higher than those reported before the advent of the iodine-thiocyanogen equations. Rose & Jamieson (117) have worked out an interesting application of these equations to the analysis of the seed oil of *Licania arborea* (Mexican), an oil which resembles oiticica oil in its drying properties. This oil contains licanic acid, a highly unsaturated C_{18} keto acid. Suitable corrections were first made for that portion of the iodine and thiocyanogen numbers due to the licanic acid; then, the residual fatty acids, oleic, linoleic, and saturated, were calculated from corrected iodine-thiocyanogen equations.

The occurrence of as much as 33 per cent of odd carbon fatty acids in the depot fats of rats and goats, fed on synthetic fats containing C_{10} to C_{23} fatty acids, was reported by Appel *et al.* (118).

Longenecker and co-workers (119, 120, 121) have described the

synthesis of a considerable series of glycerides, including β-monoglycerides, unsymmetrical monooleo-disaturated triglycerides, and dioleo-monosaturated glycerides. The reported properties of these compounds should take precedence over previously reported values, where differences exist, because of the high purity of the fatty acids used, and because of the near infallibility of the methods of synthesis. This group of investigators has adopted the numeral system of nomenclature in that they propose to thus designate the position of the fatty acid radical on the glycerol molecule as for example 1-stearyl-2,3-dicaprin. Arnold (122) described the preparation and properties of the α-lauroyl and α-caproyl esters of β-glycerophosphoric acid.

The Phosphatides

Since last year's review of the lipids (3) only a few contributions strictly related to the biochemistry of the phosphatides have appeared.

Electrometric and conductivity methods for the determination of the phosphatides of brain tissue have been described by Epshteĭn (123). Marenzi & Cardini (124) determined the phospholipid distribution in human blood plasma. Detailed analytical methods are given, the several fractions being resolved according to the following relationships:

(*a*) total phospholipid minus choline phospholipid = cephalins

(*b*) choline phospholipid minus sphingomyelin = lecithins

Sphingomyelin was determined directly. Their results in mg. per cent follow: total phospholipid, 203.7; choline phospholipid, 159.7; lecithin, 126.7; cephalin, 42.7; sphingomyelin, 35.3; and choline, 23.8. The calcium and serum protein binding powers of brain cephalin were investigated by Drinker & Zinsser (125), who found that in normal plasma 30 to 40 per cent of the bound calcium may be bound in non-diffusible form by the cephalin present.

The discovery by Folch (126) in 1942 that brain cephalin is a mixture of phosphatidyl ethanolamine, inositol, and serine has been followed by the demonstration of serine in human brain phosphatides by Schuwirth (127). Serine was isolated in the free state by extraction of the barium hydroxide hydrolyzate with butyl alcohol and also by the formation of the β-naphthalene sulfonic acid derivative.

Rewald (128) reported the lecithin and cephalin contents of the press cakes of peanuts, cottonseed, and flaxseed, and found nearly twice as much cephalin as lecithin. Thornton & Kraybill (129) extracted the phosphatides of soybean oil by adsorption with aluminum

silicate. The P/N ratio was about 1, and there was more lecithin present than cephalin. Chromatographic adsorption was ineffective in separating these phosphatides. Woolley (130) isolated a new phosphatide from soybeans to which the name lipositol was assigned. The hydrolysis products included inositol (16 per cent), galactose (15.5 per cent), and *d*-tartaric acid (8.3 per cent). He concluded that lipositol must have been a mixture of three phosphatides on the basis of the fatty acid mixture which consisted of about half oleic acid and half saturated acids. The saturated acids consisted of a mixture of 5 per cent cerebronic acid and 95 per cent of a 70 to 30 mixture of palmitic and stearic acids.

Certain aspects of the chemistry of the phospholipids have been discussed by Chaikoff (131) in the course of a review of phospholipid metabolism as studied by use of the isotope P^{32}. Chaikoff *et al.* (132) followed the phospholipid turn-over in dogs before and after injection with plasma of dogs previously treated with labelled radiophosphorus. Lorenz, Perlman & Chaikoff (133) found that radiophosphorus administered to laying hens appeared rapidly in the phospholipids of the yolk of the egg.

Additional references to the phospholipids as related to other lipids are to be found in the next section.

Tissue Lipids, Normal and Pathological

As part of a program of studying the effect of dietary factors on tissue phospholipids, Artom & Fishman (134) have set up analytical procedures for the determination of total lipids and phospholipids in animal tissues. The procedure provides for the approximate evaluation of total lipids, non-choline and choline phospholipids, sphingomyelins, non-phospholipid fatty acids, and unsaponifiable material in small samples of tissue. In rats raised to about 125 gm. on an adequate diet, total lipids, choline, and non-choline phospholipids of liver and muscle were remarkably constant when expressed on the basis of 1 gm. of lipid-free tissue. Non-phospholipid fatty acids and unsaponifiable material were more variable. On diets containing 5, 10, and 30 per cent of casein (135), there was a marked decrease in the choline phospholipids of the liver, and no change in the phospholipid composition of muscle. On the 5 and 10 per cent casein diets neutral fat accumulated in both muscle and liver, but on 30 per cent casein this accumulation failed to appear. Supplementation of the diet with cho-

line, ethanolamine, and other substances did not restore the phospholipid composition of the liver (136).

The occurrence of aldehyde lipids in tissues was reported by Feulgen & Behrens (137) in 1928. These aldehydes were liberated from the tissues by oxidation or hydrolysis and were called "plasmal." Gomori (138), employing Bennett's histochemical test, found large amounts of such lipids in adrenal cortex. Anchel & Waelsch (139) have developed methods for the isolation of tissue aldehydes, the reagents being *p*-carboxyphenylhydrazine and carboxymethoxylamine. The lipid aldehydes of beef and rat muscle and brain were isolated in amounts corresponding to 0.05 to 0.2 per cent, and, from the chemical analysis, were believed to be mainly stearyl and palmityl aldehydes. Möchel (140) tested the depot fats of the rat, cat, mouse, and man for the presence of aldehydes. The fats of the adult animal reacted negatively to the "plasmal reaction" (Schiff's reagent). However, in fats deposited in very young or fetal animals, the reaction was positive; also, during demobilization of depot fat in disease or starvation, it was positive in proportion to the rate of demobilization.

The presence of lipoproteins in blood serum has been pointed out by Macheboeuf & Tayeau (141), who showed that, when sodium or potassium soaps are added to serum, lipids are set free, the fatty acids of the soaps replacing the lipids. The detached lipids are extractable with ether. In the presence of soap, ether extracts 80 to 90 per cent of the lipids. The extracted lipid is believed to have been combined with the globulin fraction, and the unextractable lipid with the albumin. McFarlane (142) observed that the lipids of serum frozen below $-25°$ can be extracted with ether. Since the β-globulin fraction (by electrophoresis) is lowered by this treatment, it is assumed that the lipid was combined with this globulin. The association between the protein and the lipid was thus destroyed by freezing.

Brückner (143), employing his orcinol reaction (144), found 14.2 to 24.4 mg. per cent of cerebrosides in human whole blood, all these being found in the red cells. Thomas (145) has noted a remarkable hyperlipemia in a nephrotic case (four and a half year old child) in which the total lipids amounted to 6.6 gm. and total cholesterol to 2.0 gm. per 100 cc. of blood. Peters & Man (146) reported the variations in the serum lipids of man under normal conditions and in thyroid (147) and kidney (148) disease.

The relative amounts of saturated and unsaturated fatty acids in the lipids of the liver, as affected by various food fats, were reported

by Channon *et al.* (149). On high fat diets, low in choline, fatty infiltration is related to the proportion of C_{14} to C_{18} saturated fatty acids. The distribution of elaidic acid in the liver and carcass demonstrated a normal fatty acid metabolism of this acid when it is included in the diet. Chaikoff *et al.* (150) followed the formation of phospholipid in the hepatectomized dog with P^{32}. Radiophosphorus was injected into normal and hepatectomized dogs and the recovery as phospholipid P^{32} was compared. Excision of the liver reduced the recovery of P^{32} in plasma, which suggests that plasma phospholipid is formed in the liver. On the other hand, labelled phospholipid of the kidney and intestine was not affected by liver removal.

The half-life periods of liver and depot fatty acids in normal mice were found to be from 2.6 to 2.8 days and from 5 to 6 days, respectively (151). However, Salcedo & Stetten (152) found that in the obese mouse the half-life period of the depot fatty acids was much longer, suggesting that in obesity there is a restriction in the rate of fatty acid oxidation.

Artom & Swanson (153) fed rats on the ethyl esters of various dibromo fatty acids. The highest degree of liver infiltration occurred when 9,10-dibromostearic acid was fed. Infiltration decreased in the following order with 13,14-dibromobehenate, 6,7-stearate, and 2,3-stearate. In each instance, a great deal of bromine was removed from the fatty acid before deposition.

An enzyme system has been prepared from pig liver which oxidizes saturated fatty acids of less than nine carbon atoms (154). Higher fatty acids are not oxidized by this system.

The lipids of the human brain were investigated as to variations with age and sex (155). Thus Weil & Liebert found that the cephalin fraction in both gray and white matter increases with age at the expense of the other lipid fractions. Adult female brain contains more cephalin than male brain, while the latter is relatively richer in the lecithins, galactolipids, and sphingomyelins. The lecithin and cephalin fractions of the female brain are richer in phosphorus than those of the male brain. Schuwirth (156) reported the following analysis of human spinal cord: water, 75 per cent; fatty substance and cholesterol, 14.8 to 19.7 per cent of the dry matter; ether-soluble glycerol phosphatides, 19.1 to 29.5; sphingomyelin, 2.6 to 2.8; cerebrosides, 5.1 to 6.2; and gangliosides, 0.0 to 0.03 gm. per 100 gm. dry substance.

Broda (157) found that visual purple solutions of the retina contain amounts of phospholipid comparable to the protein present, and

suggested the occurrence of a lipid-globulin. Reiser (158) analyzed the intestinal mucosa of swine during fasting and after a high fat meal (cottonseed oil). Phospholipid, ester and total cholesterol, fatty acids, and fat were determined. The only value to change after the fat meal was the free fatty acid content which almost doubled in from two to five hours. The implications of this finding on the mechanism of fat absorption were discussed.

The phospholipid-free fat from atheromatous intimal tissue (159) of the aorta gave after saponification, the following percentages of fatty acids: stearic, 2.9; palmitic, 14.6; oleic, 65.2; linoleic, 9.4; and arachidonic, 2.1. The role of lipids in atherosclerosis has been reviewed by Hirsch & Weinhouse (160). They conclude that these pathological lipids constitute a non-selective deposition of plasma lipids.

In anemia the total bone marrow lipid of cats decreases by half at the expense of neutral fat (161). Free fatty acid, phospholipid, and cholesterol increase under this condition.

Detailed fatty acid analyses by the ester fractionation method of the total carcass fatty acids of three groups of rats were made by Visscher & Corley (162): (*a*) low fat diet; (*b*) low fat diet plus 5 per cent palmitic acid; (*c*) same as (*b*) plus linoleic acid supplement. No great differences were shown for the three groups. The results on the low fat control diet were as follows, expressed in moles per cent: lauric, 0.1; myristic, 2.6; palmitic, 29.0; stearic, 4.0; tetradecenoic, 0.5; hexadecenoic, 13.3; octadecenoic, 49.0; linoleic, 0.1; and C_{20} unsaturated, 1.3. On diets (*b*) and (*c*) the values for linoleic acid were 0.7 and 1.4 moles per cent respectively.

Anderson and co-workers (163) examined the lipids of human lung tissue for phthiocol and tuberculostearic and phthioic acids and found none of these substances present, although, when they were incorporated in the tissue *in vitro*, they were detectable. They concluded, therefore, that these substances are not destroyed by the tissue. Anderson (164) has reviewed his extensive researches on the lipids of the tubercle bacillus. He concludes in part:

The lipids of the acid-fast bacilli are built on an entirely different pattern from the lipids occurring in ordinary plant and animal material. They also contain new and specific chemical compounds, such as fatty acids, higher alcohols, and carbohydrates. The fats are not glycerides but fatty acid esters of the disaccharide trehalose. The phosphatides are combined with a carbohydrate which contains phosphorus and which yields mannose and inositol on hydrolysis. The waxes are mixtures consisting mainly of fatty acid esters of carbohydrates, but

some true waxes and small amounts of glycerides are present in some of the wax fractions. The waxes contain certain characteristic higher alcohols. All of the wax fractions contain optically active hydroxy acids of high molecular weight, the mycolic acids.

During fertilization of sea-urchin eggs, there is a decrease of 17 per cent in phosphatide content and of 25 per cent in free cholesterol (165).

The muscle of rats on a vitamin-E deficient diet (166) contained more cholesterol and somewhat less total lipid. In this condition the free cholesterol of the brain was increased. While in the normal rat brain, the ester cholesterol is 35 per cent of the total, in the vitamin-E deficient animal this value falls to 5 per cent of the total.

One of the most comprehensive of recent investigations on tissue lipids is by Kaucher *et al.* (167) who have compared the patterns of the lipid constituents of beef organs and muscles, of muscles of other animals, and of avian and reptilian eggs. Their data include values for phospholipid, cerebroside, free and ester cholesterol, neutral fat, total lipid, and "essential lipid," this last value being total lipid minus neutral fat.

The essential lipid concentration of the various tissues is related to the extent and variety of their physiological activities and confirms a similar relationship previously demonstrated for the phospholipids, which comprise the largest fraction of the essential lipid in all the tissues studied.

Cerebroside concentration was unexpectedly high in cardiac and skeletal muscles.

LITERATURE CITED

1. Bloor, W. R., *Biochemistry of the Fatty Acids and Their Compounds, the Lipids* (Reinhold Publishing Corp., New York, 1943)
2. Hilditch, T. P., *Ann. Rev. Biochem.*, **11**, 77–102 (1942)
3. Thannhauser, S. J., and Schmidt, G., *Ann. Rev. Biochem.*, **12**, 233–50 (1943)
4. Hilditch, T. P., *The Chemical Constitution of the Natural Fats* (John Wiley & Sons, New York, 1940)
5. Jamieson, G. S., *Vegetable Fats and Oils*, 2nd Ed. (Reinhold Publishing Corp., New York, 1943)
6. Piskur, M. M., *Oil & Soap*, **20**, 36–46, 58–71 (1943)
7. Rieman, W., III, *Ind. Eng. Chem., Anal. Ed.*, **15**, 325–26 (1943)
8. Von Mikusch, J. D., and Frazier, C., *Ind. Eng. Chem., Anal. Ed.*, **13**, 782–89 (1941)

9. VON MIKUSCH, J. D., AND FRAZIER, C., *Ind. Eng. Chem., Anal. Ed.,* **15,** 109–13 (1943)
10. NORRIS, F. A., AND BUSWELL, R. J., *Ind. Eng. Chem., Anal. Ed.,* **15,** 258–59 (1943)
11. MCKINNEY, R. S., HALBROOK, N. J., AND ROSE, W. G., *Oil & Soap,* **19,** 141–43 (1942)
12. KAUFMANN, H. P., KING, B. W., AND HUANG, L. S., *Ber. deut. chem. Ges.,* **75B,** 1201–14 (1942)
13. KAUFMANN, H. P., AND GROSSE-OETRINGHAUS, H., *Fette u. Seifen,* **49,** 194–200 (1942)
14. KASS, J. P., LUNDBERG, W. O., AND BURR, G. O., *Oil & Soap,* **17,** 50–53 (1940)
15. HILDITCH, T. P., AND MURTI, K. S., *Analyst,* **65,** 437–46 (1940)
16. RIEMENSCHNEIDER, R. W., AND WHEELER, D. H., *Oil & Soap,* **16,** 219–21 (1939)
17. MATTHEWS, N. L., BRODE, W. R., AND BROWN, J. B., *Oil & Soap,* **18,** 182–87 (1941)
18. RIEMENSCHNEIDER, R. W., SWIFT, C. E., AND SANDO, C. E., *Oil & Soap,* **18,** 203–6 (1941)
19. PAINTER, E. P., AND NESBITT, L. L., *Ind. Eng. Chem., Anal. Ed.,* **15,** 123–28 (1943)
20. MITCHELL, J. H., Jr., AND KRAYBILL, H. R., *J. Am. Chem. Soc.,* **64,** 988–94 (1942)
21. MITCHELL, J. H., JR., KRAYBILL, H. R., AND ZSCHEILE, F. P., *Ind. Eng. Chem., Anal. Ed.,* **15,** 1–3 (1943)
22. BARNES, R. B., LIDDEL, U., AND WILLIAMS, V. Z., *Ind. Eng. Chem., Anal. Ed.,* **15,** 659–709 (1943)
23. KAUFMANN, H. P., *French Patents,* 853,065–66 (1940); *Chem. Abstracts,* **36,** 2436 (1942)
24. CASSIDY, H. G., *J. Am. Chem. Soc.,* **63,** 2735–39 (1941)
25. GRAFF, M. M., AND SKAU, E. L., *Ind. Eng. Chem., Anal. Ed.,* **15,** 340–41 (1943)
26. LEHRMAN, L., *J. Am. Chem. Soc.,* **64,** 2144–46 (1942)
27. KAUFMANN, H. P., *Fette u. Seifen,* **47,** 460–62 (1940)
28. CLAESSON, S., *Arkiv. Kemi, Mineral. Geol.,* **15A,** No. 9, 1–9 (1942)
29. WALKER, F. T., AND MILLS, M. R., *J. Soc. Chem. Ind.,* **62,** 106–9 (1943)
30. SWIFT, C. E., ROSE, W. G., AND JAMIESON, G. S., *Oil & Soap,* **20,** 249–50 (1943)
31. FITELSON, J., *J. Assoc. Official Agr. Chem.,* **26,** 506–11 (1943)
32. SCHUETTE, H. A., AND VOGEL, H. A., *Fette u. Seifen,* **48,** 368–69 (1941)
33. SCHUETTE, H. A., CHRISTENSON, R. M., AND VOGEL, H. A., *Oil & Soap,* **20,** 263–65 (1943)
34. DAVIS, D. S., *Ind. Eng. Chem.,* **35,** 105 (1943)
35. DAVIS, D. S., *Ind. Eng. Chem.,* **35,** 1302 (1943)
36. HOERR, C. W., POOL, W. O., AND RALSTON, A. W., *Oil & Soap,* **19,** 126–30 (1942)
37. TODD, S. S., *Oil & Soap,* **20,** 205–8 (1943)
38. KLENK, E., AND SCHUWIRTH, K., *Z. physiol. Chem.,* **267,** 260–63 (1941)

39. Schuwirth, K., *Z. physiol. Chem.*, **277**, 147–58 (1943)
40. Inoue, Y., Yukawa, H., and Katumata, H., *J. Agr. Chem. Soc. Japan*, **17**, 491–93 (1941); *Chem. Abstracts*, **36**, 4362 (1942)
41. Inoue, Y., and Yukawa, H., *J. Agr. Chem. Soc. Japan*, **17**, 771–75 (1941); *Chem. Abstracts*, **36**, 4803 (1942)
42. Brown, J. B., and Stoner, G. G., *J. Am. Chem. Soc.*, **59**, 3–6 (1937)
43. Earle, F. R., and Milner, R. T., *Oil & Soap*, **17**, 106–8 (1940)
44. Anthony, D. S., Quackenbush, F. W., and Steenbock, H., *Oil & Soap*, **20**, 53–55 (1943)
45. Cramer, D. L., and Brown, J. B., *J. Biol. Chem.*, **151**, 427–38 (1943)
46. Stetten, D., Jr., and Grail, G. F., *Ind. Eng. Chem., Anal. Ed.*, **15**, 300 (1943)
47. Farmer, E. H., *Trans. Faraday Soc.*, **38** (1942); *Chem. Abstracts*, **36**, 5678 (1942)
48. Kapp, R., and Knoll, A., *J. Am. Chem. Soc.*, **65**, 2062–64 (1943)
49. Bömer, A., and Stather, J., *Fette u. Seifen*, **49**, 243–53 (1942)
50. Kass, J. P., and Radlove, S. B., *J. Am. Chem. Soc.*, **64**, 2253–57 (1942)
51. King, G., *J. Chem. Soc.*, 387–91 (1942)
52. King, G., *J. Chem. Soc.*, 37–38 (1943)
53. Hilditch, T. P., and Plimmer, H., *J. Chem. Soc.*, 204–6 (1942)
54. Atherton, D., and Hilditch, T. P., *J. Chem. Soc.*, 204–8 (1943)
55. Dorée, C., and Pepper, A. C., *J. Chem. Soc.*, 477–83 (1942)
56. Henderson, J. L., and Young, H. A., *J. Phys. Chem.*, **46**, 670–84 (1942)
57. Piguleveskii, G. V., and Artamonov, P. A., *J. Gen. Chem. (U.S.S.R.)*, **12**, 510–16 (1942); *Chem. Abstracts*, **37**, 2716 (1943)
58. Frankel, J. F., and Brown, J. B., *J. Am. Chem. Soc.*, **65**, 415–18 (1943)
59. Frankel, J. F., Stoneburner, W., and Brown, J. B., *J. Am. Chem. Soc.*, **65**, 259–62 (1943)
60. Frankel, J. F., and Brown, J. B., *J. Am. Chem. Soc.*, **63**, 1483 (1941)
61. Matthews, N. L., Brode, W. R., and Brown, J. B., *J. Am. Chem. Soc.*, **63**, 1064–67 (1941)
62. Arcus, C. L., and Smedley-Maclean, I., *Biochem. J.*, **37**, 1–6 (1943)
63. Von Mikusch, J. D., *J. Am. Chem. Soc.*, **64**, 1580–82 (1942)
64. McCutcheon, J. W., *Org. Syntheses*, **22**, 75–81 (1942)
65. McCutcheon, J. W., *Org. Syntheses*, **22**, 82–86 (1942)
66. Dolby, D. E., Nunn, L. C. A., and Smedley-Maclean, I., *Biochem. J.*, **34**, 1422–26 (1940)
67. Mowry, D. T., Brode, W. R., and Brown, J. B., *J. Biol. Chem.*, **142**, 679–91 (1942)
68. Mowry, D. T., Brode, W. R., and Brown, J. B., *J. Biol. Chem.*, **142**, 671–78 (1942)
69. Norris, F. A., Rusoff, I. I., Miller, E. S., and Burr, G. O., *J. Biol. Chem.*, **147**, 273–80 (1943)
70. Millican, C., *Ph.D. Dissertation* (The Ohio State University, Columbus, Ohio, 1942)
71. Ault, W. C., Cowan, J. C., Kass, J. P., and Jackson, J. E., *Ind. Eng. Chem.*, **34**, 1120–23 (1942)
72. Bradley, T. F., and Richardson, D., *Ind. Eng. Chem.*, **34**, 237–42 (1942)

73. TUTIYA, T., *J. Chem. Soc. Japan*, **61**, 867–69 (1940); *Chem. Abstracts*, **37**, 382 (1943)
74. TUTIYA, T., *J. Chem. Soc. Japan*, **61**, 1188–91 (1940); **62**, 10–12 (1941); **62**, 552–54 (1941); *Chem. Abstracts*, **37**, 1382–84 (1943)
75. SWERN, D., STIRTON, A. J., TURER, J., AND WELLS, P. A., *Oil & Soap*, **20**, 224–26 (1943)
76. RANDLE, S. B., *J. Assoc. Official Agr. Chem.*, **25**, 864–67 (1942); **26**, 340–46 (1943)
77. SCHALL, E. D., AND THORNTON, M. H., *J. Assoc. Official Agr. Chem.*, **26**, 404–7 (1943)
78. HARRISON, R. W., *J. Assoc. Official Agr. Chem.*, **25**, 877–86 (1942)
79. GROSSFELD, J., *Fette u. Seifen*, **48**, 355–59 (1941)
80. SCHLOEMER, A., AND RAUCH, K., *Z. Untersuch. Lebensm.*, **83**, 289–305 (1942)
81. VAHLTEICH, H. W., *Chem. Eng. News*, **21**, 1238–41 (1943)
82. HANDSCHUMAKER, E., THOMPSON, S. W., AND MCINTYRE, J. E., *Oil & Soap*, **20**, 133–34 (1943)
83. ANDREWS, J. T. R., AND RICHARDSON, A. S., *Oil & Soap*, **20**, 90–94 (1943)
84. BAILEY, A. E., FEUGE, R. O., KRAEMER, E. A., AND BAUER, S. T., *Oil & Soap*, **20**, 129–32 (1943)
85. KAUFMANN, H. P., AND KIRSCH, P., *Fette u. Seifen*, **49**, 841–54 (1942)
86. HILDITCH, T. P., AND MADDISON, L., *J. Soc. Chem. Ind.*, **61**, 169–73 (1942)
87. BALDWIN, W. H., AND PARKS, L. E., *Oil & Soap*, **20**, 101–4 (1943)
88. BULL, W. C., AND WHEELER, D. H., *Oil & Soap*, **20**, 137–41 (1943)
89. KLEINSMITH, A. W., AND KRAYBILL, H. R., *Ind. Eng. Chem.*, **35**, 674–76 (1943)
90. HILDITCH, T. P., AND MEARA, M. L., *J. Soc. Chem. Ind.*, **61**, 117–25 (1942)
91. HILDITCH, T. P., AND JASPERSON, H., *Biochem. J.*, **37**, 238–43 (1943)
92. HILDITCH, T. P., AND ZAKY, Y. A. H., *J. Soc. Chem. Ind.*, **61**, 34–36 (1942)
93. HILDITCH, T. P., SIME, I. C., AND MADDISON, L., *Biochem. J.*, **36**, 98–109 (1942)
94. LOVERN, J. A., *Dept. Sci. Ind. Research (Brit.) Food Invest.*, Special Rept. No. 51, 72 pp. (1942)
95. ZEISSET, A., AND GROSSFELD, J., *Z. Untersuch. Lebensm.*, **83**, 385–99 (1942)
96. SCHMIDT-NIELSEN, S., AND ESPELI, A., *Kgl. Norske Videnskab. Selskabs, Forh.*, **14**, 17–20 (1941); *Chem. Abstracts*, **37**, 3157 (1943)
97. ARSENJO, C. F., AND GOYCO, J. A., *Oil & Soap*, **19**, 129–30 (1942)
98. SCHUETTE, H. A., PINES, A. N., AND KREUGER, G. J., *Oil & Soap*, **20**, 158 (1943)
99. RANKOV, G., AND POPOV, A., *Fette u. Seifen*, **48**, 489–91 (1941)
100. ROEDL, G. F., AND THORNTON, M. H., *Oil & Soap*, **19**, 153–56 (1942)
101. JAMIESON, G. S., AND ROSE, W. G., *Oil & Soap*, **19**, 33–35 (1942)
102. VIDYARTHI, N. L., *J. Indian Chem. Soc.*, **20**, 45–50 (1943)
103. ARSENJO, C. F., AND GOYCO, J. A., *J. Am. Chem. Soc.*, **65**, 208–9 (1943)
104. ARSENJO, C. F., AND GOYCO, J. A., *J. Am. Chem. Soc.*, **65**, 1417–18 (1943)

105. Rasmussen, R. A., Morgal, P. N., and Miller, E. J., *Oil & Soap*, **20**, 159–61 (1943)
106. Schmidt-Nielsen, S., and Espeli, A., *Kgl. Norske Videnskab. Selskabs, Forh.*, **14**, 13–16 (1941)
107. Viollier, R., and Iselin, E., *Mitt. Lebensm. Hyg.*, **32**, 197–202 (1941)
108. Nutter, M. K., Lockhart, E. E., and Harris, R. S., *Oil & Soap*, **20**, 231–34 (1943)
109. Rankoff, G., *Fette u. Seifen*, **48**, 294–99 (1941)
110. Steger, A., and van Loon, J., *Rec. trav. chim.*, **61**, 120–22 (1942)
111. Thaler, H., and Groseff, *Fette u. Seifen*, **49**, 508–11 (1942)
112. Kaufmann, H. P., *Fette u. Seifen*, **48**, 193–95 (1941)
113. Tutiya, T., *J. Chem. Soc. Japan*, **62**, 286–87 (1941)
114. Steger, A., and van Loon, J., *Rec. trav. chim.*, **61**, 123–26 (1942)
115. Pritzker, J., and Jungkunz, R., *Pharm. Acta Helv.*, **17**, 69–72 (1942)
116. Painter, E. P., and Nesbitt, L. L., *Oil & Soap*, **20**, 208–11 (1943)
117. Rose, W. G., and Jamieson, G. S., *Oil & Soap*, **20**, 227–31 (1943)
118. Appel, H., Böhm, H., Keil, W., and Schiller, G., *Z. physiol. chem.*, **274**, 186–205 (1942)
119. Daubert, B. F., Fricke, H. H., and Longenecker, H. E., *J. Am. Chem. Soc.*, **65**, 1718–20 (1943)
120. Daubert, B. F., Fricke, H. H., and Longenecker, H. E., *J. Am. Chem. Soc.*, **65**, 2142–43 (1943)
121. Daubert, B. F., Spiegl, C. J., and Longenecker, H. E., *J. Am. Chem. Soc.*, **65**, 2144–46 (1943)
122. Arnold, H., *Ber. deut. chem. Ges.*, **74B**, 1736–40 (1941)
123. Epshteĭn, Y. A., *Biokhimiya*, **7**, 68–78 (1942)
124. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, **147**, 371–78 (1943)
125. Drinker, N., and Zinsser, H. H., *J. Biol. Chem.*, **148**, 187–96 (1943)
126. Folch, J., *J. Biol. Chem.*, **146**, 31–33, 35–44 (1942)
127. Schuwirth, K., *Z. physiol. Chem.*, **277**, 87–96 (1942)
128. Rewald, B., *Biochem. J.*, **36**, 822–24 (1942)
129. Thornton, M. H., and Kraybill, H. R., *Ind. Eng. Chem.*, **34**, 625–28 (1942)
130. Woolley, D. W., *J. Biol. Chem.*, **147**, 581–92 (1943)
131. Chaikoff, I. L., *Physiol. Revs.*, **22**, 291–317 (1942)
132. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333–40 (1943)
133. Lorenz, F. W., Perlman, I., and Chaikoff, I. L., *Am. J. Physiol.*, **138**, 318–27 (1943)
134. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405–14 (1943)
135. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 415–22 (1943)
136. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 423–30 (1943)
137. Feulgen, R., and Behrens, M., *Z. physiol. Chem.*, **256**, 15–20 (1938)
138. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **51**, 133–34 (1942)
139. Anchel, M., and Waelsch, H., *J. Biol. Chem.*, **145**, 605–14 (1942)
140. Möchel, G., *Z. physiol. Chem.*, **277**, 135–46 (1943)
141. Macheboeuf, M. A., and Tayeau, F., *Bull. soc. chim. biol.*, **23**, 31–48, 49–61 (1941)

142. McFarlane, A. S., *Nature,* **149**, 439 (1942)
143. Brückner, J., *Z. physiol. Chem.,* **268**, 251–56 (1941)
144. Brückner, J., *Z. physiol. Chem.,* **268**, 163–70 (1941)
145. Thomas, E. M., *Am. J. Diseases Children,* **65**, 770–75 (1943)
146. Peters, J. P., and Man, E. B., *J. Clin. Investigation,* **22**, 707–14 (1943)
147. Peters, J. P., and Man, E. B., *J. Clin. Investigation,* **22**, 715–20 (1943)
148. Peters, J. P., and Man, E. B., *J. Clin. Investigation,* **22**, 721–26 (1943)
149. Channon, H. J., Hanson, S. W. F., and Loizides, P. A., *Biochem. J.,* **36**, 214–20 (1942)
150. Fishler, M. C., Entenman, C. E., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.,* **150**, 47–55 (1943)
151. Stetten, D., Jr., and Grail, G. F., *J. Biol. Chem.,* **148**, 509–15 (1943)
152. Salcedo, J., Jr., and Stetten, B., Jr., *J. Biol. Chem.,* **151**, 413–16 (1943)
153. Artom, C., and Swanson, M., *J. Biol. Chem.,* **148**, 633–39 (1943)
154. Munoz, J. M., and Leloir, L. F., *J. Biol. Chem.,* **147**, 355–62 (1943)
155. Weil, A., and Liebert, E., *Quart. Bull. Northwestern Univ. Med. School,* **17**, 117–20 (1943); Weil, A., *Growth,* **7**, 257–64 (1943)
156. Schuwirth, K., *Z. physiol. Chem.,* **278**, 1–6 (1943)
157. Broda, E. E., *Biochem. J.,* **35**, 960–66 (1941)
158. Reiser, R., *J. Biol. Chem.,* **143**, 109–14 (1942)
159. McArthur, C. S., *Biochem. J.,* **36**, 559–70 (1942)
160. Hirsch, E. F., and Weinhouse, S., *Physiol. Revs.,* **23**, 185–202 (1943)
161. Krause, B. F., *J. Biol. Chem.,* **149**, 395–404 (1943)
162. Visscher, F. E., and Corley, R. C., *J. Biol. Chem.,* **147**, 291–95 (1943)
163. Anderson, R. J., Reeves, R. E., Creighton, M. M., and Lothrop, W. C., *Am. Rev. Tuberc.,* **48**, 65–75 (1943)
164. Anderson, R. J., *Yale J. Biol. Med.,* **15**, 311–45 (1943)
165. Ohman, L. O., *Naturwissenschaften,* **30**, 240 (1942)
166. Heinrich, M. R., and Mattill, H. A., *Proc. Soc. Exptl. Biol. Med.,* **52**, 344–46 (1943)
167. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Biochem.,* **3**, 203–15 (1943)

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS

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The present review attempts to evaluate those recent contributions to protein chemistry which bear on the problem of the structure of the intact protein molecule. Starting with consideration of the preparation and identification of purified proteins, evidence for the chemical composition is discussed in the light of methods of hydrolysis of proteins and of the determination and isolation of amino acid constituents. A hypothetical resynthesis of the protein molecule from the degradation products is reflected in the section dealing with the amino acid distribution within the native protein while the section on size, shape, and electrochemical properties deals with the intact protein in solution. Considerations of internal structure are subordinated to the limited experimental evidence available to date in support of several stimulating hypotheses. The topic of denaturation has been considered only to the extent to which it supplements a recent review on this subject (1).

The past two years have witnessed the publication of several monographs dealing with more or less specialized aspects of protein chemistry. Of these, the widest in scope is an addendum to Schmidt's *Chemistry of Amino Acids and Proteins* (2) which brings the first edition up to date. The electrophoresis of proteins has been reviewed by Abramson, Moyer & Gorin (3). Certain aspects of the physical chemistry of proteins have been considered in great detail in a monograph by Cohn, Edsall, and co-workers (4). A textbook on *Physical Biochemistry* by Bull (5) deals with several fundamental problems in the field, important for advanced students and research workers alike.

As far as fundamental investigations are concerned, the past year has been rather lean. Attention has been focused primarily on the application of well established methods to more or less practical problems, particularly in the fields of immunochemistry (6, 7) and medical research in general. Despite their importance, they can not be considered here. The reviewers have been guided in the selection of papers

by the general theme of protein structure and even within this limitation, they have felt free to omit references that did not appear to be relevant to the ideas they wished to express.

PREPARATION AND IDENTIFICATION OF PROTEINS

During the past two years, a large part of the published work in this field has been devoted to the preparation of proteins possessing unique and specific biological properties, i.e., enzymes, hormones, viruses, and immune bodies. Although notable advances have been made in the isolation of these specific proteins in relatively pure form, the methods used possess by this time little novelty. The time-honored use of ammonium sulfate, of alcohol or acetone, and of isoelectric precipitation or separation, by centrifugation and electrophoresis respectively, still remain the chief tools of the protein chemist. The particular case of the preparation of the serum proteins has been adequately considered in Volume XII of the *Annual Review of Biochemistry* and will not be discussed in this section except to note that preliminary studies have been made on the use of detergents as precipitants for the separation of these and other proteins (8, 9).

Enzymes

Myokinase. — This acid-stable protein, which occurs in skeletal muscle, has been purified by fractionation with strong acids (10). The enzymatic properties are preserved in remarkable fashion even after the protein has been heated with strong hydrochloric acid at 90° C. The enzyme catalyzes the reversible reaction between adenosinediphosphate on the one side and adenosinetriphosphate and adenylic acid on the other. The enzymatic properties are diminished by treatment with hydrogen peroxide but are fully restored by addition of sulfhydryl compounds.

Phosphorylase. — This enzyme, which catalyzes the reaction between glycogen, inorganic phosphate, and glucose-1-phosphate, has been obtained as a crystalline protein from rabbit muscle, and its catalytic properties have been extensively studied (11 to 14). The method of obtaining the specific protein consists essentially in an ammonium sulfate precipitation of the dialyzed muscle extract from which some protein had been earlier removed at pH 5.8; the precipitated protein is dialyzed in the cold against a cysteine-glycerophosphate buffer at pH 6.8. The protein crystals possess the properties of a euglobulin,

and have a molecular weight of about 400,000. The protein contains a prosthetic group of which adenylic acid is at least one component; the latter may be removed by an enzyme present in muscle (in the protein fraction removed at pH 5.8). Addition of adenylic acid to the protein component restores the enzymatic activity. An interesting point in the preparation of the crystalline material is the necessary presence of cysteine as a solubility factor.

"Pseudo" cholinesterase.—An albumin has been isolated from the pancreas which catalyzes the hydrolysis not only of choline esters but of a wide variety of fatty acid esters as well; it is obtained from this tissue by a series of ammonium sulfate precipitations interspersed by an adsorption on infusorial earth (15).

Zymohexase. — This enzyme which catalyzes the splitting at a carbon-carbon bond of hexoses into trioses (16) has been obtained as a crystalline protein by successive fractionations with ammonium sulfate of an acetone-precipitate of rat muscle extract. From the collected skeletal muscle tissue of twenty to thirty rats, about one gram of the crystals is obtained.

"Fermentation enzyme." — Practically identical crystalline proteins have been isolated respectively from rat muscle and from the Jensen rat sarcoma, which catalyze the reaction between pyruvic acid and dihydropyridine nucleotide on the one side and lactic acid and oxidized pyridine nucleotide on the other (17).

There are some interesting features in the preparation of this protein, namely (*a*) the use of yeast nucleic acid as a precipitating agent and the removal of the nucleic acid in a subsequent step with protamine, and (*b*) the final isolation of the protein as a crystalline mercury salt from an ammoniacal ammonium sulfate buffer. The crystalline mercury-protein is catalytically inactive but may be rendered completely active by dialysis against a potassium cyanide solution; the reaction is reversible. In this connection the recent study of mercury complexes of the proteins is of some interest (18).

Invertase.—Methods have been described for the purification of this enzyme from brewers' and bakers' yeast, involving (*a*) bentonite as an adsorbent, (*b*) precipitation with specific acids of high anion binding capacity (such as picric, picrolonic, flavianic, and nucleic acids), and (*c*) salting-out with ammonium sulfate (19). Five highly purified invertase preparations have been compared in their catalytic behavior toward twenty-eight different carbohydrate substrates (20).

Cytochrome oxidase.—Improvements in the method of prepara-

tion have been made by (*a*) thorough mechanical destruction of the tissue, and (*b*) exposing the enzyme suspension to ultrasonic radiation and subsequent high speed centrifugation, both operations increasing the yield of a soluble enzyme preparation of high specific activity (21).

Tyrosinase.—Further purification of this enzyme has been accomplished by fractional precipitation with ammonium sulfate (22).

Hormones

The protein hormones have been reviewed in Volume XII of the *Annual Review of Biochemistry* and elsewhere (22a), and only the more recent developments, insofar as they illustrate clear-cut or unusual protein methods, need be mentioned here.

Prolactin and other pituitary hormone fractions have been separated by a new procedure involving preliminary shaking of the macerated pituitary tissues with chloroform. On centrifugation, three liquid layers are obtained (23): the bottom layer contains mostly lipid, the central layer is a chloroform-water gel containing most of the tissue proteins plus prolactin and the adrenotropic hormones, while the top layer is a clear aqueous solution of the gonadotropic hormones, thyrotropin, pituitrin, etc. Prolactin is removed from the gel by acid-methanol. The latter procedure is based on the discovery that this hormone is soluble at pH 1 to 4.7 in 99.8 per cent methanol or in 95 per cent ethanol (24). To what extent the presence of adventitious impurities is responsible for the alcohol solubility of this as well as the other pituitary proteins (24) is not clear.

The preparation, purification, and study of the properties of the pituitary protein responsible for the adrenotropic effect of the gland have been announced independently by two laboratories with entirely concordant findings (25, 26). Both groups describe the protein as homogeneous, as lacking in sulfhydryl groups, of molecular weight about 20,000, and with an isoelectric point of 4.7. The hormone protein is apparently stable at 100° C. at pH 7.5 (26). Methods for the isolation and purification of intermedin, a hormone arising from the intermediate lobe of the pituitary gland, have been described (27). The stability of the active principle toward the rather drastic treatments involved, as well as its ready dialysis through cellophane membrane, suggest intermedin to be a polypeptide rather than a protein.

Chromatographic adsorption on permutit and elution with alco-

holic ammonium acetate have been employed for the preparation and purification of chorionic gonadotropin of pregnancy urine (28).

Purification of the growth hormone of the anterior pituitary has been effected by a method of isoelectric precipitation of the active globulin after reduction of the latter with cysteine (29).

Ferritin

An interesting series of studies on this unusual protein (30) has been reported during the past year (31 to 36). The protein may be obtained as a crystalline cadmium salt, containing over 20 per cent of iron, and inhomogeneous as to size although homogeneous electrophoretically. The iron may be removed completely from the protein by reducing the former to the ferrous state and by subsequent dialysis in the presence of bipyridine (apoferritin). The regeneration of ferritin can be accomplished only by mixing apoferritin with the brown mother liquor remaining after crystallization of the ferritin, for the iron in ferritin consists of micelles of colloidal ferric hydroxide of unusual magnetic state. Ferric iron may possess 1, 3, or 5 unpaired electrons in the outer shell; in ferritin (33), and possibly in ferric hemoglobin (37), in ferric cytochrome-*c* (38), and perhaps even in catalase (39), the number of such unpaired electrons is 3. The animal body appears capable of converting ordinary ferric iron of 5 unpaired electrons (as in ferric ammonium citrate) to the 3 electron form (33). X-ray powder diagrams of ferritin and of apoferritin show nearly identical structures, suggesting that the inherent structure of the protein is little affected by the presence or absence of the relatively large amount of iron (34).

PROTEIN HYDROLYSIS

The effectiveness of any hydrolytic agent can be measured in general terms by (*a*) the relative amount of amino nitrogen liberated (40, 41), (*b*) the relative amount of carbon dioxide liberated by ninhydrin (40), (*c*) the titer of alkali in the presence of formaldehyde (42), (*d*) the relative amount of ammonia liberated (41, 42), and (*e*) the increase in non-precipitable nitrogen by the use of protein precipitating agents (42).

For any given protein, the rate of protein hydrolysis by boiling with concentrated, completely dissociated acids is largely independent of the nature of the anion of the acid (42). At lower temperatures

and level of acidity (i.e., 65 to 75° C. and 0.05 M), the nature of the anion becomes of considerable importance, and the effectiveness of any member of a group of totally dissociated acids, all at the same concentration and pH, tends to parallel the affinity of its anion for the protein (42). Under these conditions, for example, cetylsulfonic acid is about 100 times more effective in hydrolyzing amide bonds than is hydrochloric acid. Thus the anion functions catalytically, and is concerned with "promoting the combination of hydrogen ions with these weakly basic groups [amide and peptide] . . . ." (42). The concept that the anion of the effective acid combines with the protein is supported by the stoichiometric correspondence between the amounts of anion which suffice to give the maximum rate of hydrolysis and the number of basic groups in the protein.[1] The catalytic function of the anion decreases with increasing acidity and temperature, and hence under the usual procedures of hydrolyzing proteins, the effect of the anions becomes negligible. These important experiments, while of little immediate practical value, are of interest because they suggest the possible mechanisms underlying the action of the proteolytic enzymes. The concept that the enzymes function by first combining with their substrates is long familiar.

Proteins may also be partially hydrolyzed under submaximal conditions, i.e., with acids at low temperatures (40, 46), or with crystalline or purified enzymes known to halt their activity at certain stages (47, 48). The intermediate split products of digestion, if not greater than tetrapeptides in size, may be characterized by the relative ratio of increase in carbon dioxide by ninhydrin to increase in amino nitrogen, after complete hydrolysis of the products; the ratios are 2 for the dipeptides, 1.5 for the tripeptides, and 1.33 for the tetrapeptides (40). The presence of proline and hydroxyproline obviously interferes with this kind of analysis. Treatment of wool and of edestin with concentrated hydrochloric acid at 37° C. for six days yielded largely a mixture of tripeptides (40). The possibility of the formation of artefacts cannot, however, be overlooked in all procedures of partial hydrolysis.

Certain of the keratins, because of their insolubility, are hydrolyzed with difficulty, and hence some kind of dispersion medium must be employed. The preliminary cleavage of the disulfide bonds in the keratin has appeared to be a necessary condition for such dispersion,

[1] The physical combination of the anions of the long, hydrocarbon chain type with proteins has been demonstrated by many investigators (8, 9, 43, 44, 45).

and the employment of such reducing agents as thioglycollate has been found to be useful (49, 50). The reduced protein, however, is soluble only in alkali, a circumstance not generally favorable because of the secondary reactions which alkali may bring about (50). Reduction and dispersion may nevertheless be simultaneously effected by employing neutral thioglycollate as the reducing agent and various denaturing agents, of which guanidine hydrochloride is the most effective, as the dispersing agent (50a). No dispersion occurs in the absence of the reducing agent. The use of the denaturants permits dispersion in neutral solutions. Detergents combine with the keratins (50a) as well as with many other types of protein (see footnote 1), and the secondary reactions of which they are apparently capable (42) must be closely observed.

ISOLATION AND DETERMINATION OF AMINO ACIDS

The Hydrolyzed Protein

For purposes of quantitative estimation, the amino acids have been generally divided into three categories depending upon their essential electrochemical structure, namely (*a*) the hexone bases, (*b*) the dicarboxylic acids, and (*c*) the monoamino monocarboxylic acids. The separation of these three classes has been accomplished by various procedures, of which the most successful have been for the hexone bases the use of phosphotungstic acid as a precipitant (51, 52), electrolytic transport in solutions of defined pH (53, 54), and the use of permutit (55), activated fuller's earth (56), alumina (57), and resins (58) as selective adsorbents. Although chromatographic analysis has been recently proposed (59), the best method for separating the dicarboxylic acid fraction is still the Foreman procedure whereby insoluble calcium salts of these acids are formed in dilute alcohol (60, 61). This procedure has been the subject of highly searching investigations (60, 61), to some extent as a result of the curious claims of Kögl *et al.*, relative to the presence of *d*-glutamic acid in tumors (62). Significant improvements in the Foreman method with resulting higher yields of the dicarboxylic acids have been obtained by the removal of cystine as the cuprous mercaptide from the hydrolysate prior to the addition of lime, and by the reworking of the lime-ethanol filtrates (61). Failure to remove the cystine before addition of the alkaline lime results in partial dismutation of the cystine to form insoluble calcium salts which interfere with the subsequent determination of aspartic acid. The ele-

gant procedure of Chibnall *et al.* (61) may be briefly summarized as follows: first, the separation from the hydrolysate of cystine as the cuprous mercaptide, second, the precipitation of the dicarboxylic acids by the Foreman procedure, and third the precipitation of the hexone bases as the phosphotungstic acid complexes; these procedures, performed consecutively, leave only the monoamino monocarboxylic acids in the final filtrate.

In the case of the hexone bases, histidine, arginine, and lysine have been separately estimated by electrolytic transport in solvents of different pH (54), by differential treatment with alkali and nitrous acid (51), and by the differential solubility of their silver salts at various pH values (63). The last-mentioned, classical Kossel procedure, carefully improved by Vickery (64, 65), is the most accurate. For absolute identification, histidine may be isolated as the nitranilic acid (66) and 3,4-dichlorobenzenesulfonic acid (65) complexes, or else colorimetrically estimated (66, 67); arginine has been isolated as the flavianic acid complex (65, 68), or else estimated colorimetrically by the Sakaguchi reagent (67, 69); while lysine has been quantitatively isolated from the residual filtrates as the picrate salt (64).

The separate estimation of each of the known monoamino monocarboxylic acids still remains the great problem of protein analysis. Certain of the amino acids of this category can be estimated with some ease; these are the acids which contain, in addition to the α-amino and α-carboxyl groups, yet a third polar or chemically-reactive group, i.e., serine, threonine, cystine, cysteine, methionine, tyrosine, phenylalanine, and tryptophane. Specific tests for these groups have been developed, so specific indeed that they may be employed directly on the hydrolysate without interference from members of the other two classes of amino acids. Serine and threonine, when treated with periodate, yield formaldehyde and acetaldehyde quantitatively (70, 71, 72). The sum of these two acids comes very close to accounting for all of the hydroxyamino acids in those proteins in which the presence of relatively large amounts of β-hydroxyglutamic acid was earlier reported (73). Considerable doubt is therefore cast on the probability that the last-mentioned acid occurs at all in proteins, a doubt amplified by the finding that the "hydroxyglutamic acid fraction" may consist of appreciable quantities of dismutation products of cystine together with aspartic acid (60). Cystine plus cysteine may be estimated as the precipitated cuprous mercaptide (74), by the highly specific reaction of Sullivan (75), by the less specific colorimetric reaction of Folin

(76), by the iodometric procedures of Baernstein (77) and of Okuda (78), by the colorimetric method of Vassel (79, 80), and by a polarographic technique (81). The separate estimation of cystine and of cysteine by an ingenious method utilizing the different reducing capacities of cyanide and of sodium amalgam has been recently reported (82) and represents the first unequivocal proof that these amino acids may coexist within a protein; this finding will be discussed further below. Methionine has been estimated iodometrically (83, 84), colorimetrically (85, 86), and gravimetrically (87). Tyrosine has been estimated colorimetrically by the Folin (88) and Lugg (89) technique, tryptophane by the excellent Lugg method (89) and by two recent micro-colorimetric procedures (90, 91).

The remainder of the amino acids in this group, the acids with only hydrogen or non-polar hydrocarbon chains attached to them, are estimated only with great difficulty and to date with relatively little accuracy. The physical properties of these acids, glycine, proline, alanine, and the isomeric valines and leucines, overlap too much to effect ready physical separations while the side chains offer no opportunity for specific chemical reaction with added reagents.

The current interest in protein composition both from the theoretical and practical points of view has led to the development of four quite different techniques, designed for the separation and estimation not only of the unreactive monoamino monocarboxylic acids but of the polar acids as well. These are (*a*) the solubility method, (*b*) the chromatographic method, (*c*) the microbiological method, and (*d*) the isotopic dilution method. All four of these methods are at the time of writing in the developmental stages, but taken together they appear highly promising for the eventual solution of this difficult analytical problem.

(*a*) *The solubility method.*—This technique is applicable to the estimation of any substance possessing acid or basic groups which form sparingly soluble, dissociable salts (92, 93). The method requires the measurement of the solubility of a given solid phase in two aliquots of the solution under analysis. The solid phases are produced by the addition of unequal amounts of a reagent to equal aliquots which produces the precipitation of a sparingly soluble, binary salt of a specific amino acid. If the solubility products of the salts in each aliquot are equal, the solubility relations need only be equated to solve for the unknown concentration of amino acid studied. The method has been employed for the estimation of leucine and of glycine, employing as reagent for

the former 2-bromotoluene-5-sulfonic acid, and for the latter 5-nitronaphthalene-1-sulfonic acid.

(*b*) *The chromatographic method.*—Although this technique has frequently been employed with permutit, alumina, and various resins for the separation of either the hexone bases or the dicarboxylic acids from the remainder of the hydrolysate, the application to the mono-amino monocarboxylic acid fraction is quite recent. For the latter class of acids, charcoal has been chiefly employed (94, 95, 96). The adsorption on this material is roughly parallel with the length of the side chain. The column may be developed by water, whereby considerable differences between the neutral and the aromatic amino acids appear (95, 96), the former being the more readily eluted. The fraction containing the neutral amino acids may then be run through an alumina column previously treated with formaldehyde (95). Glycine and serine are adsorbed and thus separated from the non-adsorbed alanine, proline, and isomeric leucines.

Ingenious methods have been developed along this general line whereby separation of the acetylated amino acids is accomplished by differences in partition between two liquid phases of the substances to be separated, one of the liquid phases being held substantially stationary (97, 98). One of the liquid phases is water, saturated on a solid silica gel which supports it, and containing an indicator dye. The other phase which contains the acetylated amino acids is composed of various non-aqueous solvents or mixtures thereof, and flows over the aqueous silica gel. The position of the acids is revealed by the change in color of the acid-base indicator. Subsequent wash-fluids of non-aqueous solvents develop the bands. Alanine and glycine are strongly "adsorbed" at the top of the column as slow-moving bands. Separations have been effected for phenylalanine, the isomeric leucines, the isomeric valines, proline, and alanine. Further development requires the search for more selective partition systems and solvents.

(*c*) *The microbiological method.*—This technique depends upon the finding that certain amino acids are essential for the growth of micro-organisms (99 to 104). Two forms of bacteria have been used for this study, namely *Lactobacillus arabinosus 17-5* and *Lactobacillus casei*. The amino acids essential for the growth of the former are glutamic acid, tryptophane, threonine, valine, leucine, isoleucine, cystine, lysine, and phenylalanine. The determination of any one of these essential acids in a protein hydrolysate is conducted by adding the appropriate aliquot thereof to a culture of the organism whose nutritive medium

is complete save for this one amino acid. The quantitative growth response on addition of the aliquot (either by estimation of the lactic acid produced or by turbidity measurements) is a measure of the concentration of essential amino acid added. Standard growth curves for each amino acid must be known. The method is valid only for the naturally-occurring optical isomers of the amino acids, and for each organism only for those acids essential for its growth.

(*d*) *Method of isotopic analysis.*—This is conducted by adding a pure sample of a particular amino acid containing a known amount of deuterium or N^{15}, in stable position, to a protein hydrolysate (105). A sample of the same amino acid is then isolated in pure form from the hydrolysate. If the amount of added amino acid is X, and its isotope content is Co while the isotope concentration of the specimen of pure amino acid as finally isolated is C, then the amount of amino acid, Y, present in the original mixture is:

$$Y = \left(\frac{Co}{C} - 1\right) X$$

In only one of these four methods, namely that of chromatography, is the amino acid to be determined actually completely isolated. The great advantage of all four methods is that the analysis may be performed on very small amounts of protein. Certain of the methods, however, require specialized equipment not generally available.

The Intact Protein

The estimation of certain amino acids may be conducted on intact proteins without the necessity of hydrolyzing the latter. These are amino acids with reactive groups on their side chains for which specific reagents or techniques designed for detection are available. The analyses may be conducted on the intact native or on the intact denatured protein, depending upon the amino acid studied and perhaps upon the method employed. Cysteine, cystine, tyrosine, the hexone bases, and the dicarboxylic acids have been estimated in this fashion. The estimation of the amino acids in the intact protein is not recommended as a routine analytical procedure for it cannot supplant the ultimate analysis of the hydrolyzed protein; rather its value lies in the light which the results often shed both on the nature of the binding forces which hold the native protein within its unique configuration

and on the inherent structure of the native protein, information which the analysis of the hydrolyzed protein is hardly capable of supplying.

Hexone bases and dicarboxylic acids.—These amino acids may be estimated in the native protein over the pH-stability range by calculations based on the electrometric titration curves as developed by Cannan (106, 107). Analysis of the curves for each of the amino acids depends upon the fortunate circumstance that the values of the dissociation constant for the free titratable groups of the dicarboxylic acids, of histidine, of lysine, and of arginine, are sufficiently separated so that each may be readily distinguished. The position of histidine is further characterized by the intermediate value of the heat of ionization for the imidazole group, and that of lysine by the considerable acid shift of certain segments of the titration curve within certain pH limits following the addition of formaldehyde.

The methods of Cannan are ingenious and fortunately are suitable for studies on the native protein, a feature confirmed by the reversibility of the titration curve over the studied interval between pH 2 and 10 (107). From the titration curves of egg albumin and of β-lactoglobulin, the equivalents of the dicarboxylic acids and of histidine and of arginine check rather closely with the corresponding values found in the hydrolyzed proteins (108). The number of amino groups found by titration in both proteins, however, exceed that number which would be furnished by all the ε-amino groups of the known lysine residues (108). That this discrepancy may be real is supported by the concordance of the amino values estimated in the titrated protein with those found after Van Slyke analysis. The excess of titratable amino groups in the proteins over those due to ε-amino groups of lysine may be attributed to the presence of α-amino groups (108). It may be pointed out, however, that the estimation of the amino groups rests largely on the somewhat arbitrary choice of 8.5 as the pH of the inflection point on the titration curves, with the further assumption that at this point all amino groups are ionized.

Tyrosine.—The phenolic group of tyrosine ionizes at about a pH of 10 (109), reacts readily with the Folin phenol reagent (110), and absorbs ultraviolet light with a pronounced maximum at 2800 Å (111). Within the native protein molecule, however, the constituent tyrosine residues contribute little, if anything, to the ionization curve of the protein (112), react very little with the phenol reagent (110, 113), and absorb little ultraviolet radiation (111). Unlike the side chain groups of the hexone bases and of the dicarboxylic acids, the phenolic group

of tyrosine is unreactive in the native protein, or at least the linkages in which the latter group participates are of a firmer nature than the former. However, when the proteins are denatured, the tyrosine residues react readily with the Folin reagent (110, 114) and absorb ultraviolet radiation (111); the greater the degree of denaturation of the protein, the greater the number of reactive groups so revealed (110, 111). Indeed, the number of tyrosine residues within the fully denatured protein may be ascertained from the known extinction coefficient for the free acid and from the absorption curve of the protein in the ultraviolet region of the spectrum; in the case of insulin, the number of tyrosine groups so revealed checks closely with that found in hydrolysates of the protein (111). In the case of egg albumin (111), the complete number of tyrosine residues known to be present in the protein was not revealed by the absorption curves; it is possible that this discrepancy may have been due to the failure to employ more powerful denaturing agents than alkali or urea.

Cysteine and cystine.—The mercaptan group of cysteine and the disulfide group of cystine react readily with oxidizing and reducing agents, respectively. In the native protein these groups are little reactive; in the fully denatured protein these groups are as completely reactive as in the free amino acids (1, 115). In solutions of guanidine hydrochloride, all proteins studied are completely denatured and mercaptan groups can be quantitatively titrated with standardized oxidizing agents (1), while acid-denatured proteins may be titrated for these groups with iodine (116). Disulfide groups may be completely revealed in proteins insufficiently denatured to reveal the full number of mercaptan groups known to be present (1). The proportion of cysteine and of cystine so estimated in the intact denatured proteins has been found to be in excellent agreement with that respectively found in hydrolysates of these proteins (1, 116). The problem of the sulfur distribution in proteins has been extensively considered in a recent review on denaturation (1) and need not be considered further except to note that the sum of cysteine, cystine, and methionine sulfur is equal in the proteins studied to the total sulfur (1, table 5, 117).

AMINO ACID COMPOSITION AND DISTRIBUTION

The application of the results of amino acid analysis to the problem of protein structure has led to four notable suggestions, namely: (*a*) the 2^n3^m stoichiometric rule of Bergmann & Niemann (118); (*b*) Chib-

nall's thesis that the sub-units of the proteins may consist either of single or of multiple polypeptide chains (108); (*c*) Astbury's reconciliation of the Bergmann-Niemann generalization (119) with the noted exceptions to it (108), i.e. the rule is followed by single polypeptide chains (as in edestin) but not by a system of multiple polypeptide components (intermolecular as in egg albumin, intramolecular as in β-lactoglobulin) although each of the multiple components might itself conform to the rule; and (*d*) the nearly common amino acid pattern in homologous proteins from widely-differing species (120 to 126).

Examination of the available analytical data reveals a large number of instances in substantial agreement with the Bergmann-Niemann generalization, and it would appear that this rule, while perhaps of limited validity, comes very close to representing the fundamental stoichiometry of individual polypeptide systems within proteins. The subsidiary generalization, namely that relating to the sequence of the acids within the polypeptide chain, is not so readily tested and requires the isolation and identification of partially hydrolyzed products of chain length sufficient to contain the necessary number of residues, followed by the identification of the order of the residues in these polypeptide fragments. The pattern of the individual chains, however, may not necessarily be based upon sequences of single but rather of groups of residues; in this sense the similar physical properties of proteins possessing quite different compositions, such as myosin and wool, may be rendered explicable (119).

Patterns of nearly similar amino acid composition appear to occur in tissue proteins of the same type. The myosins (120), the whole muscle proteins (125), the liver nucleoproteins (123), the keratins (121), and the neuroproteins (121), each from a number of different species, appear to have nearly the same proportion of several amino acids.

Not only the animal but also the homologous plant proteins share this property of general similarity, for the relative uniformity of amino acid composition has been pointed out in the case of the somatic, photosynthesizing tissues of related cryptogams (124). Individual small differences in composition unquestionably exist between members of each homologous group (65, 123), and without minimizing the importance of such differences, the over-all similarities appear to be worthy of particular interest as illustrating a nearly common pattern for the proteins of each group (127).

PHYSICAL PROPERTIES OF PROTEINS

The topics of size, shape, and electrochemical properties of protein molecules have been so adequately discussed in a preceding volume of this series (128) and elsewhere (4, 129, 130, 131), that this discussion shall be limited to matters of interpretation and to certain aspects of the relation between these properties and protein structure in general.

While molecular size and shape are useful and important physical criteria for the characterization of native proteins, *per se* they reflect but little of the specific chemical structure and biological activity of a protein. This is borne out by inspection of available data (131) which show proteins of widely different origin, function, and stability to share with each other similar, if not identical, molecular-kinetic properties. It is therefore not surprising that sedimentation and diffusion measurements may fail to resolve protein mixtures into their component parts (131, 132, 133). While the fundamental work of Svedberg and his associates (131) has been one of the most revolutionary developments in protein chemistry, the widespread application of their methods to purified preparations can yield little more than an accumulation of additional data unless such studies are correlated with other sources of evidence. Similarly, estimation of molecular shapes, as based on viscosity (134, 135, 136), diffusion (137, 138), dielectric dispersion (4, 130, 139, 140), or birefringence methods (141), remains more of academic interest unless interpretation is made in the light of other known properties of the protein in question. Considerations of size and shape assume prime importance when applied to an investigation of the changes in these properties occurring under well-controlled external conditions and it is here that the methods under consideration reveal their full merits (cf. 1, 131, 142).

Molecular Weight

Sedimentation.—Practical aspects of ultracentrifugal analysis of proteins have been reviewed by Pickels (143). The result of a recent conference on the theory and practice of sedimentation analysis has been made the subject of a valuable publication (144). It has also been shown that with large spherical molecules, the molecular weight may be estimated with a fair degree of accuracy by sedimentation in the angle centrifuge, the addition of sucrose or other non-sedimenting materials serving to maintain the density gradient (145). The effect

of concentration on the speed of sedimentation of suspensions of spherical (146) and asymmetric (147) particles has been considered.

Molecular weight analyses have been reported for ferritin and apoferritin (148), crystalline muscle phosphorylase (11), crystalline carbonic anhydrase (149), prolactin (150, 151), the parathyroid hormone (152), adrenocorticotropic hormone (26), the soluble LS antigen of vaccinia (153), and for the M protein of hemolytic streptococci (154). In a study relating the sedimentation constant of the infectious principle of tobacco mosaic virus to that of the paracrystalline nucleoprotein (155), it was observed that within a probable error of $\pm$ 6 per cent, the two are in agreement.

Investigation of unreduced and reduced insulin in the presence of an anionic detergent (Duponol PC) yielded for the unreduced protein-detergent complex a molecular weight of 27,000 and an estimated value of about 9,000 for the protein component, in contrast to 46,000 for the native insulin in aqueous buffers (43). Preparations of insulin in which disulfide linkages were progressively reduced by increased amounts of thioglycollic acid were found to be increasingly aggregated in aqueous solutions, while in the presence of Duponol, the sedimentation constant was considerably lower than that of the native protein (43). Though the authors favor the interpretation that the detergent micelles exert a dissociating effect on the protein molecule, the possibility is also considered that the charge effect of the anionic detergent micelles caused a retardation of the sedimentation and diffusion rate of the protein without changing its molecular weight.[2] The molecular characteristics of gluten dispersed in sodium salicylate were studied by McCalla & Gralén (156).

Following previous studies on the effects of certain enzymes on the

[2] When the values for observed sedimentation constant of both unreduced and reduced insulin are plotted against the concentration of Duponol, a linear relation obtains below Duponol concentrations of 1 per cent for the unreduced proteins, and between 2 and 0.5 per cent Duponol for the reduced protein, yielding an extrapolated value of $S = 4.70$ for the former, and of about $S = 3$ for the latter, as compared to $S = 3.55$ for the native protein. Conceivably, these values may represent the sedimentation constants of the proteins in the presence of detergent, after elimination of the charge effects of the detergent micelles. While diffusion measurements are lacking, one may interpret the higher value to indicate aggregation of the unreduced insulin molecules, the lower value a decrease in molecular weight or an increase in frictional ratio of the reduced insulin. Further investigation of this phenomenon would be highly desirable.

splitting of antibody globulins, Petermann (156a) found beef serum pseudoglobulin and diphtheria antitoxin to be split by papain into fragments of one half and one quarter of the original molecular weight. Since the sedimentation characteristics of the remaining globulin were found to be unchanged it was concluded that enzyme action had proceeded without prior "denaturation" of the whole molecules, although previous work from the same laboratory had shown certain denatured proteins to sediment with the same rate as the parent native protein (173). It is also debatable whether the change in configuration required for the enzyme to act on a few structure-essential bonds could be detected by sedimentation measurements (cf. 1).

The classical studies by Svedberg and associates (131), and of Brohult (142) on the dissociation of the large hemocyanin molecules have been supplemented recently by similar investigations on tobacco mosaic virus protein (157). Preliminary data reveal that in alkaline buffers, the virus dissociates into two components, one of them free of nucleic acid, and both having a molecular weight of about 360,000, as compared to about 40,000,000 for the intact virus protein. Readjustment of the solutions containing either one of the biologically inactive dissociation products to pH 5 yielded a material practically indistinguishable from the native protein in molecular weight and shape, in crystal form and electrophoresis, but biologically inactive.

Diffusion.—The investigation of proteins by diffusion measurements has been the subject of a comprehensive review (138). The effects of solvent composition, solute concentration, and heterodispersity have been considered as have the application of combined diffusion and viscosity measurements to the estimation of molecular weights. Previous work from the same laboratory had already demonstrated the practical usefulness of these combined methods when applied to proteins of varying molecular weight and shape (135). Such studies have recently been extended to preparations of native and regenerated bovine serum albumin (158). Again, satisfactory agreement with sedimentation and diffusion data was found.

Other methods.—As a result of experimental improvements (159 to 164) osmotic pressure measurements have once again come to the fore. Equilibrium is attained within a short period of time. Molecular weights are obtained by various methods of extrapolation to zero concentration (165). Strict monodispersity is a prerequisite for obtaining true molecular weights since the presence of a small percentage (by weight) of low molecular weight impurities has a large effect on

the number average molecular weight as obtained by osmotic pressure methods (165). Measurements have been reported for irradiated serum proteins (161) and for mixtures of serum or serum albumin with sodium thymonucleate (166). The latter study revealed that in such mixtures thymonucleate is osmotically inactive, probably as a result of suppression of dissociation accompanied by aggregation.

The method of light scattering has been applied recently to the estimation of molecular weights. By using a protein of known molecular weight as a standard, the unknown molecular weight may be estimated (167, 168, 169). Good agreement with the accepted values has been obtained for several purified proteins (168, 169).

The small angle x-ray diffraction of colloidal solutions is determined by the size and shape of the dispersed particles. Application of this method to chymotrypsin yielded values in reasonable agreement with the known diameter of these protein molecules (170).

Molecular Shape

The concept of specific shape characteristics of protein molecules is a rather recent development and originated from the thesis that the frictional coefficient effective in diffusion, sedimentation, or viscosity is higher than that calculated for spherical molecules, even if reasonable allowance for hydration is made (129, 134, 171). After it was shown what sort of asymmetries would result for a number of representative proteins and their dissociation products if hydration were to be neglected (129, 134, 171, 172, 173), this method of interpretation has been extended to additional data (cf. 130, 135, 174, 175, 176), with allowance for varying, assumed degrees of hydration. An analogous analysis of dielectric dispersion data is due to Williams *et al.* (139, 177) and has since been extended and elaborated by Oncley (cf. 4, 130). The problem has been reviewed on several occasions (*vide infra*) and its complexity does not warrant detailed consideration within the space of the present review. However, since certain criticisms have been voiced in some quarters (cf. 178, 179), while in others the estimated values for molecular shape have been taken too literally (180), several explanatory remarks may be in place.

The term "apparent" molecular shape was designed to apply to asymmetries calculated on the basis of zero hydration (171), though it was never implied that protein molecules are unhydrated (129, 135). Further limitations were recognized to arise from approximations involved in the hydrodynamic approach to the theories of viscosity and

translational diffusion, and from uncertainties regarding the actual shape of protein molecules (171). While these shortcomings do not invalidate the concept of distinct shape characteristics of protein molecules, it has been obvious that estimation of molecular shapes cannot be better than the theories on which the methods of measurement are based. Although it has not yet been possible to determine unambiguously the degree of hydration, various lines of reasoning suggest that it hardly exceeds about 0.3 gm. of water per gm. of anhydrous protein (129, 130, 135, 181). In an interesting analysis of the problem Bull & Cooper (182) arrived at an empirical relation between volume intrinsic viscosities and diffusion constants from which the volume hydration of dissolved proteins is calculated (28.3 ± 4.2 per cent). However, the question remains how much significance can be attached to an empirical relation admittedly derived from equations which themselves are only first approximations. Other limitations are discussed in the paper just cited.

Interpretation of x-ray diagrams of crystalline hemoglobin (page 141) led to the conclusion that these protein molecules are essentially impenetrable to the solvent molecules, and that the water of hydration is confined to one molecular layer. If this should be found to be generally true, hydration would play a secondary role in the calculation of molecular shapes, not only for the more asymmetric but also for the larger protein molecules (129).

Several lines of experimental approach have provided additional evidence for the non-spherical shape of proteins. X-ray studies have yielded data for the over-all dimensions of several purified proteins (cf. 183). Measurements of the double refraction of flow of myosin, tobacco mosaic virus, fibrinogen, hemocyanin, and others, have furnished a fair estimate for the length of these molecules (quoted in 141). Lastly, while as yet limited to the large virus proteins (184, 185, 186), and myosin (187), electron microscope examination of dried specimens has shown some of these proteins to be highly asymmetric while others approximate a more nearly spherical shape.[3] A comprehensive review of the size and shape of viruses has been published by Markham *et al.* (188).

While an evaluation of the full significance of molecular shape characteristics in terms of protein structure has to await further developments, a functional relation between internal structure and external

[3] Attempts to extend such studies to smaller protein molecules yielded inconclusive results (184).

shape cannot be doubted. This is clearly illustrated by the regular shape changes accompanying dissociation of certain proteins into smaller units (134, 171, 173) and by the profound changes occurring under the influence of more powerful denaturing agents (cf. 1).

Electrophoresis

Recent improvements in experimental technique have rendered this method of particular value for the characterization of purified proteins in terms of homogeneity and net charge (3), and for the analysis of protein mixtures, particularly of plasma and serum. The latest advances in the investigation of proteins by Tiselius' moving boundary method have been excellently reviewed by Longsworth (189). Simple experimental apparatus for preparative purposes has been described by Svensson (190) and by Hahn & Tiselius (191).

Conditions which a homogeneous and pure protein has to satisfy are (*a*) that it migrate with a single electrophoretic boundary over the entire pH-stability range and (*b*) that the extent of boundary spread be wholly accounted for by the diffusion occurring within the time of migration. In this connection it is of interest to note that all preparations of crystalline horse serum albumin that have been studied thus far in that manner, although monodisperse in sedimentation and diffusion, and in electrophoresis on the alkaline side of the isoelectric point, reveal boundary splitting and boundary asymmetries when subjected to electrolysis below pH 6 (192). The situation is somewhat analogous to that observed with crystalline egg albumin, singly (193) or in mixtures with nucleic acid (194), and suggests that even the most carefully prepared, crystalline, carbohydrate-free serum albumin is a mixture. A similar situation has been observed with globins from various species prepared by the method of Anson & Mirsky by denaturation with acids, followed by regeneration (195, 196). Here, too, the isolated protein is electrophoretically monodisperse between pH 5.2 and 7.9, while in more acidic buffers, two boundaries appear in a mass ratio of about 6:4. When separated from each other by electrophoretic means, the two components are found to differ from each other in acid-binding capacity, and sulfur content (196).

While a homogeneous protein has to satisfy the condition of electrophoretic homogeneity, the converse of this statement is far from true. Thus, serum γ-globulin, when isolated electrophoretically or by fractionation with salts (197) or ethanol (198) exhibits a single elec-

trophoretic boundary over a wide pH range, whereas it is known to fail to satisfy the requirement of constant solubility. It is therefore surprising that Jameson & Alvarez-Tostado (199) claimed to have isolated from horse serum a γ-globulin of constant solubility (as judged by a method not commonly employed) but heterodisperse in electrophoresis.

Electrophoretic analyses have been reported also for heat-inactivated pneumococcal antibody (200), prolactin (150, 151), tuberculin proteins (201), bovine serum albumin (158), the renin activator (serum α_2-globulin?) (202) and for egg albumin detergent mixtures (44) (see also page 144).

Changes in electrophoretic patterns have been recorded for human serum and its fractions following irradiation with ultraviolet light, leading to an increased homogeneity (161) similar to that obtained after heat treatment (203). The latter changes are inhibited by saturation of plasma with glucose (204). Addition of 2.8 M urea to normal human serum gives rise to several additional boundaries, the jagged pattern being due to convection rather than to changes in component distribution (205). Regeneration of serum albumin, following denaturation by concentrated urea, yields a protein which is only slightly less homogeneous electrophoretically than the native material, the mobility being increased by several per cent (158). Marked changes in pattern and mobility have been noted following heat-treatment of crystalline horse serum albumin (206).

INTERNAL STRUCTURE

The problem of the internal arrangement of polypeptide chains within the protein molecule has continued to stimulate both experimentation and imagination. It is here that all aspects of protein chemistry come to meet, for any specific structure assigned to a protein has to be compatible with all evidence that may be brought to bear, whether the latter be derived from amino acid analyses, from physico-chemical investigations of the intact, denatured, or chemically modified proteins, or from determinations of specific biological activity. The difficulties attending the determination of the internal structure of proteins are considerable: experimentally, because the standard diffraction methods designed for determination of the molecular structure of simpler organic molecules, when applied to proteins, fail to yield a

comparable degree of resolution; theoretically, because the distribution of the amino acid side chains may profoundly influence the configuration of polypeptide chains (119, 207 to 210).

Fibrous Proteins

Astbury's classical investigation and interpretation of the x-ray diffraction of the keratins, collagens, and myosin have been reviewed extensively here (212, 213) and elsewhere (119, 211). In the past year, an attempt was made to correlate certain proposed structures with the Bergmann-Niemann hypothesis of the periodicity of occurrence of amino acid residues (119). While x-ray data do not seem to support the view that the residues follow one another exactly in the periods suggested by their relative proportions, the idea is expressed that a broad stoichiometry of types of residues is maintained in conformity to a common molecular plan. Thus, a molecular scheme is proposed for the keratin-myosin group according to which side chains are packed in triads which occur alternately on one and the other side of the plane of the main chains (214). The side chains of which a triad is composed are believed to be essentially either all polar or all non-polar, salt-like linkages between ionized groups, and hydrogen bonds between amide and hydroxyl, or between hydroxyl and hydroxyl groups, forcing the polar groups of one or adjacent chains into such combinations. While this scheme need not be strictly adhered to—indeed it would require an even distribution between polar and non-polar amino acid residues—it is thought to explain the noted long-range elasticity of proteins of the keratin and myosin group, and, unlike earlier structures, it is compatible with the space requirements of the side chains. However, while these ideas are fully as ingenious as those by which they were preceded, they too are attended by a high degree of ambiguity, corresponding to, and necessitated by the rather incomplete data from which they are derived.

The widespread belief in a structural pattern common to certain classes of fibrous proteins has led to an attempt to short-cut the search by excluding those configurations which are incompatible with certain general principles and tentative assumptions (215). Reasoning from these, models of fully extended polypeptide chains have been constructed in which the chains are held together by hydrogen bonds between nitrogen and carbonyl oxygen atoms. While all these structures appear to be in good agreement with the limited experimental data, and in accord with reasonable assumptions, their number is

admittedly too large to warrant any single structure to be favored or accepted.

Proteins share with other high polymeric substances the characteristic property of being composed of sub-units of like structure and property, and accordingly, they may be expected to adhere to a structural scheme determined by the recurrence of identical groupings along the chains. However, this analogy is not quite complete since the structure-determining influence of the peptide groups may be counterbalanced by profound variations in nature and distribution of the amino acid side chains. Since stereochemical considerations reveal considerable differences in the space requirements of individual amino acid residues (207, 216, 217), it is obvious that the general principle of "close-packing" would demand a different configuration of polypeptide chains in regions of shorter and smaller side chains, than in regions of the longer and larger ones; and unless the side chains are distributed according to size and shape—a hypothesis devoid of any experimental evidence—the arrangement of the polypeptide chains will be attended by a certain element of disorder. Another, and possibly even more important structure-determining factor is introduced by the attractive forces operating between ionized and polar side chains (210), thereby creating a specificity of structure in response to the attractive forces that are exerted by the multitude of amino acid side chains. While in the face of characteristic diffraction periods a certain regularity of structure cannot be gainsaid, it seems that in the past, the structure-determining role of the side chains hardly has been accorded proper recognition. While the exact structure-determining role of the various side chains cannot yet be evaluated, in certain instances reasonable deductions have been made. Thus, it has been shown that the presence of glycine residues may confer upon a polypeptide chain a considerable degree of internal freedom, thereby allowing the chain to assume configurations in response to the attractive forces exerted by other, more reactive side chains (209). Similar reasoning has led to the belief that in a polypeptide chain, the large proline and hydroxyproline residues have to occupy terminal positions in order to be accommodated (218), a hypothesis which need not necessarily be maintained if these amino acids hold positions adjacent to glycine (209).

Electrostatic attraction between the ionized end groups of glutamic and aspartic acid on one hand, and of the hexone bases on the other, have long been considered as a potential source of interaction between various parts of one, or between adjacent polypeptide chains (cf. 219),

as have hydrogen bonds between polar groups such as those of serine (108, 220) or threonine.

There is one instance where a considerable body of evidence has become available concerning the role of amino acid residues in fibrous proteins, i.e., that of cystine. It was suggested earlier by Astbury (221), Speakman (222), and their collaborators that a number of important physical and chemical properties of wool keratin are associated with the presence of disulfide linkages of the cystine moiety, which serve to connect adjacent polypeptide chains. In a more recent series of investigations, Harris and co-workers found (50, 223, 224) that cleavage of these linkages by reduction with thioglycollic acid in neutral or acid solutions, while leaving the gross fiber structure intact, greatly increased extensibility and alkali solubility and reduced tensile strength. Alkylation of the reduced wool with aliphatic dihalides introduced hydrocarbon chains between pairs of sulfur atoms, and led to the restoration of the physical properties of the original wool keratin, in respect to both elasticity and tensile strength. The conclusion most pertinent to the present discussion is that the long-range elasticity of the natural fibers has to do with the strain imposed by the cystine cross linkages on the otherwise more flexible and extended polypeptide chains, forcing the latter to contract to a more random and probable state.

From methylation studies of native and reduced wool the conclusion has been drawn that "activated" peptide linkages are capable of condensation with hydroxyl and sulfhydryl groups of serine and cysteine, respectively, causing ring closures of the oxazoline and thiazoline type (225).

The x-ray diffraction properties of several fibrous proteins have been investigated during the past year. Redetermination of fiber photographs of keratins of porcupine quill revealed a spacing of 5.14 Å along the fiber axis, and a large unit of pattern of 658 Å or 198 Å (226). The latter value appears to be correct according to independent researches (227). Lateral spacings of 81, 41, and about 9.8 Å have been noted, the latter being considerably resolved, containing some twenty discrete reflections. A total of more than one hundred reflections have now been established in the range of 1 to 150 Å (226).

Preliminary investigations of human fibrinogen and fibrin suggest both proteins to resemble in x-ray and elastic properties natural myosin and keratin (228). Thus, fibrinogen-fibrin conversion does not appear to be associated with profound structural changes.

A long diffraction period of 640 Å observed with collagenous tissue (229) has been identified in electron microscope photographs of the intact tissue as the average distance between transverse bands (230, 231). New small-angle interferences of 33, 42, and 66 Å have been reported for myosin (232).

Corpuscular Proteins

The problem of the application of x-ray methods to the determination of the unit cell dimensions and symmetry of protein crystals has been excellently reviewed in several places (119, 178, 183). A deeper insight into the fine structure of protein molecules has been obtained by the examination of electron density projections of protein crystals at different stages of shrinkage of the wet crystals (179). The degree of optical resolution was found to be enhanced when crystallization of horse methemoglobin was effected by electrodialysis rather than by salting-out with ammonium sulfate (233). In agreement with earlier work (179), it was found that the hemoglobin molecules form coherent sheets parallel to the c-plane, with layers of mother liquor between the protein sheets. It has now been established that the layers of water of crystallization are dispersed intermolecularly rather than intramolecularly, i.e., the liquid lies between protein layers one molecule thick rather than between layers of half-molecules. The authors also established the existence of four roughly equal and equidistant peaks in the protein portion of the electron density projection, little less than 9 Å apart. Of the various interpretations with which this finding is compatible, they chose one in terms of which the hemoglobin molecule consists of four equal and parallel layers of polypeptide chains, with the main chains folded in the plane of the layers and with the side chains at right angles to it. Further, it is suggested that polar side chains occupy the outside surface of the layer structure, the non-polar ones being tucked away in the interior six surfaces. The over-all dimensions of the air-dried crystal are 36 Å x 48 Å x 64 Å, corresponding to an axial ratio of about 2 : 1.

The idea of a layer structure of native corpuscular protein was proposed several years ago by Astbury (234) and arose in part from Gorter's investigation of the structure of protein monolayers (235). It has been revived by Chibnall in the interpretation of his recent analytical work (108). As discussed in a preceding section (page 128), he concluded that with certain proteins, such as edestin, β-lactoglobu-

lin, egg albumin, and insulin, the number of ionizable groups as determined by amino acid analysis can be reconciled with that adduced from titration data, only if these protein molecules are assumed to consist of more than one polypeptide chain (see also 210), varying from four for egg albumin to as many as eighteen for insulin.

While for certain proteins the idea of an association of sub-units appears to be corroborated by molecular weight determination on native and denatured material, with others no such evidence is available (1). Thus, hemoglobin, edestin, and a variety of other corpuscular proteins are known to be rather susceptible to dissociation into smaller components, whereas, for instance, egg albumin and crystalline serum albumin are notably resistant to molecular dissociation. The case of hemoglobin is of particular interest in this connection, since recent interpretation of the kinetics of acid denaturation has led to the conclusion that this reaction is initiated by an activation process, totally accountable for by the combination of one protein molecule with two protons, resulting in the cleavage of two hydrogen bonds and in the subsequent dissociation of the molecule into halves (236). A layer structure of the type suggested by the English workers is compatible with these findings.

What has been said in the preceding section about the role of side chains as structure-determining factors applies equally to the corpuscular proteins. Indeed, the contribution of these groups may be expected to be even stronger in this case since their closer geometrical proximity in a globular molecule affords more profound interactions. Conversely, separation of the side chains will be one of the first steps in denaturation, followed by an opening-up of the polypeptide chains (1, 210, 219). However, it may be in place to record a word of caution against evaluating all these hypotheses, no matter how ingenious and stimulating they may be, with less hesitancy than is called for by the limited experimental evidence from which they were derived.

X-ray diffraction measurements have recently been reported for β-lactoglobulin, obtained from a large single crystal grown from salt-free solutions by McMeekin's method (237). The cell dimensions are in good agreement with those previously obtained (178). Measurements on ferritin and apoferritin revealed for both materials the location of diffraction lines to correspond to those one would expect for a face-centered cubic cell containing 8 molecules (35). The molecular weight deduced from air dried crystals is in good agreement with the ultracentrifugal value.

DENATURATION

The problem of the denaturation of proteins has been the subject of a recent review in which the widely scattered experimental data have been critically examined, in an attempt to integrate them with present-day concepts of protein structure (1). The denaturation process has been considered therein as a truly chemical reaction, with due regard to the fact that proteins are endowed with unique and specific biological functions; accordingly, a variety of chemical, physical, and biological sources of evidence have been drawn upon in the analysis of the problem. In order to avoid duplication, we shall anticipate the ideas expressed in that review and limit the present discussion to certain aspects of the denaturation process that have been in the focus of recent investigations.

Synthetic Detergents

The interaction between the amphoteric proteins and anionic or cationic detergent micelles may involve the ionized groups of the protein as evidenced by precipitation; or it may affect the intrinsic structure of the protein molecule in an act of denaturation. In addition, a catalytic effect of detergent acids in the hydrolysis of protein amide and peptide bonds has been established (42).

Precipitation.—Precipitation has been shown to be confined to pH regions in which protein and detergent carry charges of opposite sign (8, 9, 45, 238, 239), the pH at which precipitation ceases approximating closely the isoelectric point of the protein. Precipitation is also governed by the protein-detergent weight ratio, temperature, and ionic strength (8, 239). Since precipitation by anionic detergents has been found to lead to changes in properties which may not be wholly reversed after dissociation of the protein-detergent complex, the protein so recovered must be said to have undergone some degree of denaturation, according to the general definition proposed for denaturation (1, 239).

Denaturation.—Higher concentrations of detergent than are requisite for precipitation are needed for the detergents to reveal their full denaturing effects. Previous work has shown that denaturation of proteins by detergents may be manifested in the liberation of protein thiol groups (240), the inactivation and disintegration (241, 242) or aggregation (243) of viruses, changes in absorption spectrum (244), and the dissociation of pigment-protein compounds (245, 246). Deter-

gents have also been found to be good dispersing agents for fibrous proteins (50a). Viscosity measurements revealed the denaturing action of relatively low concentrations of certain detergents to exceed that of concentrated solutions of guanidine hydrochloride, one of the most powerful denaturants hitherto known (8, 239).

In an interesting series of investigations, Lundgren *et al.* reported the practical usefulness of synthetic detergents for the formation of protein fibers of high tensile strength (44, 247, 248).

Electrophoretic analysis (44) of mixtures of egg albumin and alkyl benzene sulfonate revealed the formation of a complex in a detergent-protein weight ratio of 1:3, which is in good agreement with independent evidence (8, 239). Excess protein or detergent gives rise to additional electrophoretic boundaries characteristic of the individual components. In contrast, mixtures of heat-denatured egg albumin and detergent form complexes of continuously varying composition within the same range of detergent concentration in which the native protein maintains a constant combining ratio. This suggests that upon heat denaturation, more reactive groups become available for combination, as a result of partial unfolding of the molecule. Since in regions of detergent excess the native protein becomes denatured also, native and heat-denatured egg albumin are electrophoretically indistinguishable from each other in this region.

X-ray diffraction measurements on egg albumin denatured by alkylbenzene sulfonate or by heat, revealed spacings characteristic of denatured proteins in general, and similar to those given by disoriented β-keratin. Stretching under steam serves to sharpen the reflections, yielding a pattern of the fully extended β-keratin type (248). Similar data have been obtained independently for various globular proteins after denaturation by heat (249).

In connection with the chain structure of synthetic detergents, it is of interest to note that urea, in concentrations in which it is effective as a denaturing agent, has been shown also to form chain-like micelles, as evidenced by viscosity measurements at low shearing stresses and by a strong Tyndall effect (250). Though the mode of action of such chains on proteins remains a matter of conjecture, this parallelism of structure and behavior invites consideration.

Immunological Application

Largely in response to the exigencies of war, the past years have witnessed a revived interest in destroying the species specificity of

proteins of animal origin in order to render them suitable for parenteral administration to humans. A valuable monograph by Mudd & Thalhimer (251) describes the possible application of casein, casein digests, and bovine serum albumin, in addition to that of purified human serum albumin and hemoglobin, to blood transfusion in the treatment of shock. Other attempts, involving more drastic treatments of the protein, have been summarized by Henry (252).

The original observation that treatment of egg albumin with strong alkali reduces the antigenic activity of the protein, supposedly by virtue of "racemization" (253), has stimulated similar investigations on serum proteins. Though the work leaves something to be desired as regards both the identification of the modified protein, and the precision of immunological tests, the fact remains that alkali treatment may greatly reduce the antigenicity, as revealed by qualitative precipitin tests and by anaphylaxis (254 to 257). While the nature of the reaction is obscure, it is apparent that treatment with 0.5 or 1 *N* sodium hydroxide leads to drastic chemical and physical changes of the proteins. Most authors agree that hydrogen sulfide is liberated during the process and that, depending on the extent of treatment, the modified protein may be more or less toxic.

Experiments on both horse and beef serum albumin reveal that denaturation by urea or guanidine hydrochloride, followed by regeneration, yields a material of reduced antigenic activity but unimpaired serological specificity (258, 259). Since under the conditions of these experiments, the changes in the protein were confined primarily to its internal structure, it appears that the latter is influential, at least in part, in determining the ability of the protein to elicit antibody synthesis.

Preliminary data have been published to show that denaturation of pneumococcal horse antibody globulin by guanidine hydrochloride does not impair its ability to react *in vitro* with the homologous carbohydrate (260). Both the irreversibly denatured and the regenerated fractions were serologically active although combination with the antigen occurred in different combining ratios. These observations are not readily compatible with the alleged synthesis *in vitro* of antibodies from normal globulins, by denaturation followed by regeneration in the presence of an antigen (261). While the latter work would be of considerable theoretical and practical importance, it cannot be regarded as sufficiently conclusive to be accepted (7) as supporting evidence for a stimulating theory (218).

LITERATURE CITED

1. Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O., *Chem. Revs.*, **34** (In press)
2. Schmidt, C. L. A., *Addendum to the Chemistry of Amino Acids and Proteins* (C. C. Thomas, Springfield, Illinois, 1943)
3. Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins and the Chemistry of Cell Surfaces* (Reinhold Publishing Corporation, New York, 1942)
4. Cohn, E. J., and Edsall, J. T., *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions* (Reinhold Publishing Corporation, New York, 1943)
5. Bull, H. B., *Physical Biochemistry* (John Wiley and Son, New York, 1943)
6. Boyd, W. C., *Fundamentals of Immunochemistry* (Interscience Publishers, New York, 1943)
7. Kabat, E. A., *J. Immunol.*, **47**, 513–87 (1943)
8. Putnam, F. W., and Neurath, H., *J. Biol. Chem.*, **150**, 263–64 (1943)
9. Schmidt, K. H., *Z. physiol. Chem.*, **277**, 117–31 (1943)
10. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127–37 (1943)
11. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151**, 21–30 (1943)
12. Cori, G. T., and Green, A. A., *J. Biol. Chem.*, **151**, 31–38 (1943)
13. Cori, C. F., Cori, G. T., and Green, A. A., *J. Biol. Chem.*, **151**, 39–56 (1943)
14. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **151**, 57–63 (1943)
15. Mendel, B., and Mundell, D. B., *Biochem. J.*, **37**, 64–66 (1943)
16. Warburg, O., and Christian, W., *Biochem. Z.*, **314**, 149–51 (1943)
17. Kubowitz, F., and Ott, P., *Biochem. Z.*, **314**, 94–102 (1943)
18. Haarmann, W., *Biochem. Z.*, **314**, 1–16 (1943)
19. Adams, M., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1359–69 (1943)
20. Adams, M., Richtmyer, N. K., and Hudson, C. S., *J. Am. Chem. Soc.*, **65**, 1369–80 (1943)
21. Haas, E., *J. Biol. Chem.*, **148**, 481–93 (1943)
22. Jensen, H., and Tenenbaum, L. E., *J. Biol. Chem.*, **147**, 737–38 (1943)

22a. Van Dyke, H. B., Chow, B. F., duVigneaud, V., Fevold, H. L., Irving, G. W., Jr., Long, C. N. H., Shedlovsky, T., and White, A., *Ann. New York Acad. Sci.*, **43**, 255–426 (1943)

23. Schwenk, E., Fleischer, G. A., and Tolksdorf, S., *J. Biol. Chem.*, **147**, 535–40 (1943)
24. Fleischer, G. A., *J. Biol. Chem.*, **147**, 525–34 (1943)
25. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, **149**, 425–36 (1943)
26. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **149**, 413–24 (1943)
27. Abramowitz, A. A., Papandrea, D. N., and Hisaw, F. L., *J. Biol Chem.*, **151**, 579–86 (1943)
28. Katzmann, P. A., Godfrid, M., Cain, C. K., and Doisy, E. H., *J. Biol. Chem.*, **148**, 501–7 (1943)

29. Marx, W., Simpson, M. E., and Evans, H. M., *J. Biol Chem.*, **147**, 77–89 (1943)
30. Tria, E., *Atti accad. Italia, Rend. classe sci. Fis., Mat. Nat.*, (7), **1**, 579–89 (1940); *Chem. Abstracts*, **37**, 898 (1943)
31. Granick, S., *J. Biol. Chem.*, **146**, 451–61 (1942)
32. Granick, S., and Michaelis, L., *J. Biol. Chem.*, **147**, 91–97 (1943)
33. Michaelis, L., Coryell, C. D., and Granick, S., *J. Biol. Chem.*, **148**, 463–80 (1943)
34. Granick, S., *J. Biol. Chem.*, **149**, 157–67 (1943)
35. Fankuchen, I., *J. Biol. Chem.*, **150**, 57–58 (1943)
36. Hahn, P. F., Granick, S., Bale, W. F., and Michaelis, L., *J. Biol. Chem.*, **150**, 407–12 (1943)
37. Coryell, C. D., Stitt, F., and Pauling, L., *J. Am. Chem. Soc.*, **59**, 633–42 (1937)
38. Theorell, H., *J. Am. Chem. Soc.*, **63**, 1820–24 (1941)
39. Michaelis, L., and Granick, S., *J. Gen. Physiol.*, **25**, 325–30 (1941)
40. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 1369–87 (1941)
41. Warner, R. C., *J. Biol. Chem.*, **142**, 741–56 (1942)
42. Steinhardt, J., and Fugitt, C. H., *J. Research Natl. Bur. Standards*, **29**, 315–27 (1942)
43. Miller, G. L., and Andersson, K. J. I., *J. Biol. Chem.*, **144**, 459–64, 465–74, 475–86 (1942)
44. Lundgren, H. P., Elam, D. W., and O'Connell, R. A., *J. Biol Chem.*, **149**, 183–94 (1943)
45. McMeekin, T. L., *Federation Proc.*, **1**, pt. 2, 125 (1942)
46. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 92–102 (1943)
47. Bergmann, M., Zervas, L., and Schleich, H., *Ber. deut. chem. Ges.*, **65B**, 1747–50 (1932)
48. Waldschmidt-Leitz, E., *Monatsh.*, **66**, 357–63 (1935)
49. Goddard, D. R., and Michaelis, L., *J. Biol. Chem.*, **106**, 605–14 (1934)
50. Patterson, W. I., Geiger, W. B., Mizell, L. R., and Harris, M., *J. Research Natl. Bur. Standards*, **27**, 89–103 (1941)
50a. Jones, C. B., and Mecham, D. K., *Arch. Biochem.*, **3**, 193–98 (1943)
51. Van Slyke, D. D., *J. Biol. Chem.*, **10**, 15–24 (1911)
52. Van Slyke, D. D., Hiller, A., and Dillon, R. T., *J. Biol. Chem.*, **146**, 137–58 (1942)
53. Foster, G. L., and Schmidt, C. L. A., *J. Biol. Chem.*, **56**, 545–53 (1923)
54. Foster, G. L., and Schmidt, C. L. A., *J. Am. Chem. Soc.*, **48**, 1709–11 (1926)
55. Whitehorne, J. C., *J. Biol. Chem.*, **56**, 751–56 (1923)
56. Turba, F., *Ber. deut. chem. Ges.*, **74B**, 1829–38 (1941)
57. Wieland, T., *Z. physiol. Chem.*, **273**, 24–28 (1942)
58. Block, R. J., *Proc. Soc. Exptl. Biol. Med.*, **51**, 252–53 (1942)
59. Turba, F., and Richter, M., *Ber. deut. chem. Ges.*, **75B**, 340–47 (1942)
60. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 360–71 (1943)

61. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 372–88 (1943)
62. Kögl, F., Erxleben, H., and van Veersen, G. J., *Z. physiol Chem.*, **277**, 251–63 (1943)
63. Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, **31**, 165–76 (1900)
64. Vickery, H. B., and Block, R. J., *J. Biol. Chem.*, **93**, 105–12 (1931)
65. Vickery, H. B., *J. Biol. Chem.*, **144**, 719–30 (1942)
66. Block, R. J., *Proc. Soc. Exptl. Biol. Med.*, **37**, 580–82 (1937)
67. Macpherson, H. T., *Biochem. J.*, **36**, 59–63 (1942)
68. Vickery, H. B., *J. Biol. Chem.*, **132**, 325–42 (1940)
69. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 359–64 (1942)
70. Winnick, T., *J. Biol. Chem.*, **142**, 461–66 (1942)
71. Boyd, M. J., and Logan, M. A., *J. Biol. Chem.*, **146**, 279–88 (1942)
72. Nicolet, B. H., Shinn, L. A., and Saidel, L. J., *J. Biol. Chem.*, **142**, 609–14 (1942)
73. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **142**, 139–46 (1942)
74. Vickery, H. B., and White, A., *J. Biol. Chem.*, **99**, 701–15 (1933)
75. Sullivan, M. X., *U.S. Pub. Health Repts., No.* **1084**, 1030–56 (1926)
76. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421–28 (1922)
77. Baernstein, H. D., *J. Biol. Chem.*, **89**, 125–31 (1930)
78. Okuda, Y., *J. Biochem. (Japan)*, **5**, 217–27 (1925)
79. Vassel, B., *J. Biol. Chem.*, **140**, 323–36 (1941)
80. Mecham, D. K., *J. Biol. Chem.*, **151**, 643–45 (1943)
81. Sullivan, M. X., Hess, W. C., and Smith, E. R., *J. Biol. Chem.*, **130**, 741–44 (1939)
82. Sullivan, M. X., Hess, W. C., and Howard, H. W., *J. Biol. Chem.*, **145**, 621–24 (1942)
83. Baernstein, H. D., *J. Biol. Chem.*, **115**, 25–32 (1936)
84. Lavine, T. F., *J. Biol. Chem.*, **151**, 281–98 (1943)
85. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871–76 (1941)
86. Sofin, L. H., Rosenblum, H., and Schultz, R. C., *J. Biol. Chem.*, **147**, 557–60 (1943)
87. Beach, E. F., and Teague, D. M., *J. Biol. Chem.*, **142**, 277–84 (1942)
88. Folin, O., and Ciocalteau, V., *J. Biol. Chem.*, **73**, 627–32 (1927)
89. Lugg, J. W. H., *Biochem. J.*, **32**, 775–83 (1938)
90. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **144**, 563–64 (1942)
91. Eckert, H. W., *J. Biol. Chem.*, **148**, 205–12 (1943)
92. Moore, S., Stein, W. H., and Bergmann, M., *Chem. Revs.*, **30**, 423–35 (1942)
93. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **150**, 113–30 (1943)
94. Tiselius, A., *Advances in Colloid Sci.*, **1**, 81–93 (1942)
95. Schramm, G., and Prinosigh, J., *Ber. deut. chem. Ges.*, **76B**, 373–80 (1943)
96. Wachtel, J. L., and Cassidy, H. G., *J. Am. Chem. Soc.*, **65**, 665–68 (1943)
97. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **35**, 1358–68 (1941)

98. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 79–85 (1943)
99. Greene, R. D., and Black, A., *Proc. Soc. Exptl. Biol. Med.*, **54**, 322–24 (1943)
100. Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, **98**, 266–67 (1943)
101. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615–26 (1943)
102. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511–14 (1943)
103. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193–200 (1944)
104. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83–96 (1944)
105. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737–44 (1940)
106. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 235–41 (1936)
107. Cannan, R. K., Palmer, A. H., and Kibrick, A. C., *J. Biol. Chem.*, **142**, 803–22 (1942)
108. Chibnall, A. C., *Proc. Roy. Soc. (London) B*, **131**, 136–60 (1942)
109. Greenstein, J. P., *J. Biol. Chem.*, **101**, 603–21 (1933)
110. Miller, G. L., *J. Biol. Chem.*, **146**, 339–44, 345–50 (1942)
111. Crammer, J. L., and Neuberger, A., *Biochem. J.*, **37**, 302–10 (1943)
112. Cohn, E. J., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 8–20 (1938)
113. Herriot, R. M., *J. Gen. Physiol.*, **19**, 283–88 (1936)
114. Mirsky, A. E., and Anson, M. L., *J. Gen. Physiol.*, **19**, 451–57 (1936)
115. Greenstein, J. P., and Jenrette, W. V., *J. Biol. Chem.*, **142**, 175–80 (1942)
116. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **151**, 635–42 (1943)
117. Li, C. H., *J. Biol. Chem.*, **148**, 289–91 (1943)
118. Bergmann, M., and Niemann, C., *J. Biol. Chem.*, **118**, 301–14 (1937); **122**, 577–96 (1938)
119. Astbury, W. T., *Advances in Enzymology*, **3**, 63–108 (1943)
120. Bailey, K., *Biochem. J.*, **31**, 1406–13 (1937)
121. Block, R. J., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 79–90 (1938)
122. Claude, A., *Science*, **91**, 77–78 (1940)
123. Greenstein, J. P., and Jenrette, W. V., *J. Natl. Cancer Inst.*, **1**, 91–104 (1940)
124. Lugg, J. W. H., *Biochem. J.*, **37**, 132–37 (1943)
125. Beach, E. F., Munks, B., and Robinson, A., *J. Biol. Chem.*, **148**, 431–40 (1943)
126. Block, R. J., and Bolling, D., *Arch. Biochem.*, **3**, 217–21 (1943)
127. Greenstein, J. P., *Advances Protein Chem.*, **1** (In press)
128. Edsall, J. T., *Ann. Rev. Biochem.*, **11**, 151–82 (1942)
129. Neurath, H., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 196–207 (1938); **8**, 80–93 (1940)
130. Oncley, J. L., *Ann. New York Acad. Sci.*, **41**, 121–51 (1941); *Chem. Revs.*, **30**, 433–50 (1942)
131. Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge*, p. 369 (Oxford University Press, New York, 1940)
132. Cohn, E. J., *Chem. Revs.*, **24**, 203–29 (1939)

133. KEKWICK, R. A., AND MCFARLANE, A. S., *Ann. Rev. Biochem.*, **12**, 93–114 (1943)
134. POLSON, A., *Nature*, **137**, 740 (1936); *Kolloid-Z.*, **88**, 51–61 (1939)
135. NEURATH, H., COOPER, G. R., AND ERICKSON, J. O., *J. Biol. Chem.*, **138**, 411–36 (1941)
136. LAUFFER, M. A., *Chem. Revs.*, **31**, 561–86 (1942)
137. LAMM, O., *Nova Acta Regi. Soc. Sci. Upsaliensis*, Ser. IV, **10**, No. 6 (1937)
138. NEURATH, H., *Chem. Revs.*, **30**, 357–94 (1942)
139. WILLIAMS, J. W., AND WATSON, C. C., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 208–17 (1938)
140. ARRHENIUS, S., *Nova Acta Regi. Soc. Sci. Upsaliensis*, Ser. IV, **12**, No. 5 (1940)
141. EDSALL, J. T., in COHN, E. J., AND EDSALL, J. T., *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, p. 527 ff. (Reinhold Publishing Corporation, New York, 1943)
142. BROHULT, S., *Nova Acta Regi. Soc. Sci. Upsaliensis*, Ser. IV, **12**, No. 4 (1940)
143. PICKELS, E. G., *Chem. Revs.*, **30**, 341–55 (1942)
144. MACINNES, D. A., ARCHIBALD, W. J., BEAMS, J. W., BRIDGMAN, W. B., ROTHEN, A., AND WILLIAMS, J. W., *Ann. New York Acad. Sci.*, **43**, 173–252 (1942)
145. PICKELS, E. G., *J. Gen. Physiol.*, **26**, 341–60 (1943)
146. BURGERS, J. M., *Proc. Acad. Sci. Amsterdam*, **44**, 1045–51 (1941)
147. MOSIMANN, H., *Helv. Chim. Acta*, **26**, 61–75 (1943); *Chem. Abstracts*, **37**, 6522 (1943)
148. ROTHEM, A., cited in *J. Biol. Chem.*, **146**, 451–61 (1942); **147**, 91–97 (1943)
149. PETERMANN, M. L., AND HAKALA, N. V., *J. Biol. Chem.*, **145**, 701–5 (1942)
150. WHITE, A., BONSNES, R. W., AND LONG, C. N. H., *J. Biol. Chem.*, **143**, 447–64 (1942)
151. LI, C. H., *J. Biol. Chem.*, **146**, 633–38 (1942)
152. ROSS, W. F., AND WOOD, T. R., *J. Biol. Chem.*, **146**, 49–58 (1942)
153. SHEDLOVSKY, T., ROTHEN, A., AND SMADEL, J. E., *J. Exptl. Med.*, **77**, 155–64 (1943)
154. PAPPENHEIMER, A. M., JR., WILLIAMS, J. W., AND ZITTLE, C. A., *J. Immunol.*, **43**, 61–63 (1942)
155. LAUFFER, M. A., *J. Biol. Chem.*, **151**, 627–33 (1943)
156. MCCALLA, A. G., AND GRALÉN, N., *Can. J. Research*, **20**, 130–59 (1942)
156a. PETERMANN, M. L., *J. Biol. Chem.*, **144**, 607–16 (1942)
157. SCHRAMM, G., *Naturwissenschaften*, **31**, 94–97 (1943)
158. PUTNAM, F. W., ERICKSON, J. O., VOLKIN, E., AND NEURATH, H., *J. Gen. Physiol.*, **26**, 513–31 (1943)
159. BULL, H. B., *J. Biol. Chem.*, **137**, 143–51 (1941)
160. HEPP, O., *Z. ges. exptl. Med.*, **99**, 709–17 (1936)
161. DAVIS, B. D., HOLLAENDER, A., AND GREENSTEIN, J. P., *J. Biol. Chem.*, **146**, 667–71 (1942)

162. Fuoss, R. M., and Mead, D. J., *J. Phys. Chem.*, **47**, 59–70 (1943)
163. Simms, H. S., Zwemer, R. L., and Lowenstein, B. E., *J. Lab. Clin. Med.*, **28**, 113–18 (1942)
164. Flory, P. J., *J. Am. Chem. Soc.*, **65**, 372–82 (1943)
165. Huggins, M. L., *Ind. Eng. Chem.*, **35**, 980–86 (1943)
166. Greenstein, J. P., *J. Biol. Chem.*, **150**, 107–12 (1943)
167. Lepeschkin, W. W., *Biochem. Z.*, **309**, 254–69 (1941) ; **314**, 135–48 (1943)
168. Putzey, P., and Brosteaux, J., *Trans. Faraday Soc.*, **31**, 1314–25 (1935)
169. Putzey, P., and Brosteaux, J., *Mededeel. Kon. Vlaamsche Akad. Wetenschappen Letteren Schoone Kunsten Belgie*, **3**, 3–23 (1941) ; *Chem. Abstracts*, **37**, 4750 (1943)
170. Kratky, O., and Sekora, A., *Naturwissenschaften*, **31**, 46–47 (1943)
171. Neurath, H., *J. Am. Chem. Soc.*, **61**, 1841–44 (1939)
172. Neurath, H., and Cooper, G. R., *J. Am. Chem. Soc.*, **62**, 2248–49 (1939)
173. Lundgren, H. P., and Williams, J. W., *J. Phys. Chem.*, **43**, 989–1002 (1939)
174. Mehl, J. W., Oncley, J. L., and Simha, R., *Science*, **92**, 132–33 (1940)
175. Bull, H. B., *J. Biol. Chem.*, **133**, 39–49 (1940)
176. Wyman, J., Jr., and Ingalls, E. N., *J. Biol. Chem.*, **147**, 297–318 (1943)
177. Elliott, M. A., and Williams, J. W., *J. Am. Chem. Soc.*, **61**, 718–25 (1939)
178. Crowfoot, D., *Chem. Revs.*, **28**, 215–28 (1941)
179. Perutz, M. F., *Nature*, **149**, 491–94 (1942)
180. Mirsky, A. E., *J. Gen. Physiol.*, **24**, 725–33 (1941)
181. Bull, H. B., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 140–49 (1938)
182. Bull, H. B., and Cooper, J. A., *Am. Assoc. Adv. Sci.*, Pub. No. **21**, 150–56
183. Fankuchen, I., *Ann. New York Acad. Sci.*, **41**, 157–68 (1941)
184. Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, **146**, 25–30 (1942)
185. Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *Arch. Path.*, **36**, 167–76 (1943)
186. Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, **47**, 261–83 (1943)
187. Ardenne, M. v., and Weber, H. H., *Kolloid-Z.*, **97**, 322–25 (1941)
188. Markham, R., Smith, K. M., and Lea, D., *Parasitology*, **34**, 315–52 (1942)
189. Longsworth, L. G., *Chem. Revs.*, **30**, 323–40 (1942)
190. Svensson, H., *Arkiv Kemi Mineral. Geol.*, **B15**, 1–8 (1942) ; *Chem. Abstracts*, **37**, 4274 (1943)
191. Hahn, L., and Tiselius, A., *Biochem. Z.*, **314**, 389–90 (1943)
192. Sharp, D. G., Cooper, G. R., Erickson, J. O., and Neurath, H., *J. Biol. Chem.*, **144**, 139–47 (1942)

193. Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 2580–90 (1940)

194. Longsworth, L. G., and MacInnes, D. A., *J. Gen. Physiol.*, **25**, 507–16 (1942)

195. Munro, M. P., and Munro, F. L., *J. Biol. Chem.*, **150**, 427–32 (1943)

196. Reiner, L., Moore, D. H., Lang, E. H., and Green, M., *J. Biol. Chem.*, **146**, 583–87 (1942)

197. Sharp, D. G., Cooper, G. R., and Neurath, H., *J. Biol. Chem.*, **142**, 203–16 (1942)

198. Cohn, E. J., Luetscher, J. A., Jr., Oncley, J. L., Armstrong, S. H., Jr., and Davis, B. D., *J. Am. Chem. Soc.*, **62**, 3396–400 (1940)

199. Jameson, E., and Alvarez-Tostado, C., *J. Am. Chem. Soc.*, **65**, 459–65 (1943)

200. Krejci, L. E., Jennings, R. K., and Smith, L. D., *J. Immunol.*, **45**, 111–22 (1942)

201. Seibert, F. B., and Nelson, J. W., *Am. Rev. Tuberc.*, **47**, 66–77 (1943); *J. Am. Chem. Soc.*, **65**, 272–78 (1943).

202. Plentl, A. A., and Page, I., *J. Biol. Chem.*, **147**, 143–54 (1943)

203. Van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. L., *J. Immunol.*, **40**, 39–45 (1941)

204. Hardt, C. R., Huddleson, I. F., and Ball, C. D., *Science*, **98**, 309–10 (1943)

205. Moore, D. H., *J. Am. Chem. Soc.*, **64**, 1090–92 (1942)

206. Cooper, G. R., and Neurath, H., *J. Phys. Chem.*, **47**, 383–98 (1943)

207. Neurath, H., *J. Phys. Chem.*, **44**, 296–305 (1940)

208. Mack, E., Jr., *Ohio J. Sci.*, **41**, 183–89 (1941)

209. Neurath, H., *J. Am. Chem. Soc.*, **65**, 2039–40 (1943)

210. Bull, H. B., *Advances in Enzymology*, **1**, 1–42 (1940)

211. Astbury, W. T., *Cold Spring Harbor Symposia Quant. Biol.*, **6**, 109–21 (1938)

212. Astbury, W. T., *Ann. Rev. Biochem.*, **8**, 113–32 (1939)

213. Huggins, M., *Ann. Rev. Biochem.*, **11**, 27–50 (1942)

214. Astbury, W. T., and Bell, F., *Nature*, **147**, 696–99 (1941)

215. Huggins, M., *Chem. Revs.*, **32**, 195–218 (1943)

216. Corey, R. B., *Chem. Revs.*, **26**, 227–36 (1940)

217. Neurath, H., and Bull, H. B., *Chem. Revs.*, **23**, 391–435 (1938)

218. Pauling, L., *J. Am. Chem. Soc.*, **62**, 2643–57 (1940)

219. Eyring, H., and Stearn, A. E., *Chem. Revs.*, **24**, 253–70 (1939)

220. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 365–78 (1942)

221. Astbury, W. T., and Street, A., *Trans. Roy. Soc. (London)*, **A230**, 75–101 (1931)

222. Speakman, J. B., *Nature*, **132**, 930 (1933); **138**, 327 (1936)

223. HARRIS, M., MIZELL, L. R., AND FOURT, L., *J. Research Natl. Bur. Standards,* **29**, 73–86 (1942)
224. GEIGER, W. B., KOBAYASHI, F. F., AND HARRIS, M., *J. Research Natl. Bur. Standards,* **29**, 381–89 (1942)
225. BLACKBURN, S., MIDDLEBROOK, W. R., AND PHILLIPS, H., *Nature,* **150**, 57 (1942)
226. MACARTHUR, I., *Nature,* **152**, 38–41 (1943)
227. BEAR, R. S., *J. Am. Chem. Soc.,* **65**, 1784–85 (1943)
228. BAILEY, K., ASTBURY, W. T., AND RUDALL, K. M., *Nature,* **151**, 716 (1943)
229. BEAR, R. S., *J. Am. Chem. Soc.,* **64**, 727 (1942)
230. HALL, C. E., JAKUS, M. A., AND SCHMITT, F. O., *J. Am. Chem. Soc.,* **64**, 1234 (1942)
231. SCHMITT, F. O., HALL, C. E., AND JAKUS, M. A., *J. Cellular Comp. Physiol.,* **20**, 11–33 (1942)
232. KRATKY, O., SEKORA, A., AND WEBER, H. H., *Naturwissenschaften,* **31**, (1943)
233. BOYES-WATSON, J., AND PERUTZ, M. F., *Nature,* **151**, 714–16 (1943)
234. ASTBURY, W. T., *Nature,* **137**, 803–5 (1936)
235. GORTER, E., AND GRENDEL, F., *Proc. Acad. Sci. Amsterdam,* **32**, 770–71 (1929)
236. PUTNAM, F. W., AND NEURATH, H., *Chem. Revs.,* **34** (In press)
237. FANKUCHEN, I., *J. Am. Chem. Soc.,* **64**, 2504–5 (1942)
238. JAFFÉ, W. J., *J. Biol. Chem.,* **148**, 185–86 (1943)
239. PUTNAM, F. W., AND NEURATH, H., *J. Am. Chem. Soc.* (In press)
240. ANSON, M. L., *J. Gen. Physiol.,* **23**, 239–46 (1939)
241. SREENIVASAYA, M., AND PIRIE, N. W., *Biochem. J.,* **32**, 1707–10 (1938)
242. BAWDEN, F. C., AND PIRIE, N. W., *Biochem. J.,* **34**, 1278–92 (1940)
243. PFANKUCH, E., AND KAUSCHE, G. A., *Biochem. Z.,* **312**, 72–77 (1942)
244. KEILIN, D., AND HARTREE, E. F., *Nature,* **145**, 934 (1940)
245. KUHN, R., BIELIG, H. J., AND DANN, O., *Ber. deut. chem. Ges.,* **73B**, 1080–91 (1940)
246. SMITH, E. L., AND PICKELS, E. G., *J. Gen. Physiol.,* **24**, 753–64 (1941)
247. LUNDGREN, H. P., *J. Am. Chem. Soc.,* **63**, 2854–55 (1941)
248. PALMER, K. J., AND GALVIN, J. A., *J. Am. Chem. Soc.,* **65**, 2187–90 (1943)
249. SENTI, F. R., EDDY, C. R., AND NUTTING, G. C., *J. Am. Chem. Soc.,* **65**, 2473 (1943)
250. CLARK, R. E. D., *Nature,* **151**, 642–43 (1943)
251. MUDD, S., AND THALHIMER, W., *Blood Substitutes and Blood Transfusion* (C. C. Thomas, Springfield, 1942)
252. HENRY, J. P., *J. Exptl. Med.,* **76**, 451–76 (1942)
253. TENBROEK, C., *J. Biol. Chem.,* **17**, 369–75 (1914)

254. Davis, H. A., and Eaton, A. G., *Proc. Soc. Exptl. Biol. Med.*, **50**, 246–48 (1942)

255. Arnow, L. E., Kazal, L. A., and deFalco, R. J., *J. Biol. Chem.*, **145**, 347–48 (1942)

256. Lewis, J. H., *Science*, **98**, 371–72 (1943)

257. deFalco, R. J., Kazal, L. A., and Arnow, L. E., *Science*, **98**, 542–43 (1943)

258. Erickson, J. O., and Neurath, H., *J. Exptl. Med.*, **78**, 1–8 (1942)

259. Martin, D. S., Erickson, J. O., Putnam, F. W., and Neurath, H., *J. Gen. Physiol.*, **26**, 533–39 (1943)

260. Erickson, J. O., and Neurath, H., *Science*, **98**, 284–85 (1943)

261. Pauling, L., and Campbell, D. H., *J. Exptl. Med.*, **76**, 211–20 (1942)

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CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS[1]

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In the present review reactions of a wide variety of phosphate compounds are organized with primary regard for the fate of the phosphate radical. Inorganic phosphate may be removed from or added to an organic molecule. Organically bound phosphate may be transferred to another part of the same molecule or to a different molecule, or its position may remain fixed and the change take place in some other part of the molecule. The present interpretation is made possible by the fundamental work of Warburg, Meyerhof, Cori, and others, and the reviews of Kalckar (1, 2) and Lipmann (3) on the role of phosphate in the utilization of metabolic energy. The new material covered is that made available in the last two years. The reactions to be considered are presented in tables that pertain to the various sections.

HYDROLYTIC CLEAVAGE OF PHOSPHATE BONDS

Phosphomonoesterase

Non-specific phosphomonoesterase. — The determination of the specificity of the phosphatases and the study of their kinetics depends ultimately upon the purification of the enzymes as proteins. The intestinal alkaline phosphatase has been obtained in relatively pure form by Schmidt & Thannhauser (4) by autolysis in the presence of trypsin, precipitation by ammonium sulfate, removal of impurities by the judicious use of aluminum hydroxide, and by final precipitation in acetone. This intestinal phosphatase contains 21 per cent polysaccharide and 10 per cent nitrogen (4). The ultraviolet absorption spectrum is the same in the presence and in the absence of substrate (5). The enzyme hydrolyzes monoesters and certain diesters of phosphate, and also inorganic pyrophosphate and ATP, but does not act on phospholipids or thymonucleic acid (4). Although this is the most active prep-

[1] To conserve space, the following abbreviations will be used: ATP, ADP, AMP, for adenosinetriphosphate, adenosinediphosphate, and adenosinemonophosphate, respectively.

aration yet obtained, further purification will probably yield greater specificity. Kinetic studies reveal that the time curve is that of a first order reaction only when phenylphosphate is used as substrate. The inhibition by inorganic phosphate and pyrophosphate was confirmed.

TABLE I

HYDROLYTIC CLEAVAGE OF PHOSPHATE BONDS

Class of Enzymes	Substrate	Product	Substrates for Some Specific Enzymes
Phosphomonoesterase.....	$-\overset{\vert}{\underset{\vert}{C}}-O-\overset{\Vert}{\underset{\vert}{P}}-$	$-\overset{\vert}{\underset{\vert}{C}}OH + HO\overset{\Vert}{\underset{\vert}{P}}-$	Fructose-1,6-diphosphate Phytic acid Adenosine-5-phosphate
Phosphodiesterase.....	$-\overset{\vert}{\underset{\vert}{C}}-O-\overset{\Vert}{\underset{\vert}{P}}-O-\overset{\vert}{\underset{\vert}{C}}-$	$-\overset{\vert}{\underset{\vert}{C}}OH + HO\overset{\Vert}{\underset{\vert}{P}}-O-C-$	Ribonucleic acid Desoxyribonucleic acid
Pyrophosphatase.....	$R-O-\overset{\Vert}{\underset{\vert}{P}}-O-\overset{\Vert}{\underset{\vert}{P}}-$	$R-O-\overset{\Vert}{\underset{\vert}{P}}OH + HO\overset{\Vert}{\underset{\vert}{P}}-$	Inorganic pyrophosphate Adenosinetriphosphate

The alkaline phosphatase of the mammary gland has been prepared by the Albers method (6), which involves autolysis and fractionation in organic solvents, and has been further purified on the basis of its solubility in saturated magnesium acetate (7). The acid and alkaline phosphatases of beef kidney, prepared without autolysis, have been found in the water soluble and water insoluble protein fractions, respectively (8).

Cloetens (9) has continued his work on alkaline kidney phosphatase prepared by the Albers method. This enzyme, like the alkaline phosphatase of the intestinal mucosa (4, 10), is activated to some extent by magnesium, is not inhibited by fluoride, and is strongly inhibited by potassium cyanide or cysteine. When inactivated by divalent prolonged dialysis against buffer, the enzyme can be reactivated by magnesium, calcium, manganese, cobalt, or nickel ions. The combination of the enzyme with dilute concentrations of the metal ions requires time, and the presence of substrate inhibits the combination. If the same enzyme is dialyzed against a potassium cyanide solution for six days and the potassium cyanide removed by further dialysis, it requires two metals for reactivation, i.e., the addition of one of the above ions must be preceded by the addition of traces of divalent zinc, cobalt, or mercury ions. The inhibition of the undialyzed phosphatase by potassium cyanide is probably due to complex formation with zinc

(9). Massart & Dufait found a high zinc content in this enzyme preparation (11).

Fluoride inhibits acid phosphatase (12) and neutral yeast phosphatase. The latter enzyme, after dialysis, is reactivated by magnesium, cobalt, or manganese ions. Sodium fluoride inhibits the magnesium activated enzyme much more than the manganese or cobalt activated enzyme since the magnesium-fluoride complex is more stable (11). Beck (13) finds that while kidney phosphatase is inhibited by phlorhizin at pH 5, there is no inhibition at pH 6.9, the intracellular pH of the kidney tubule, and he concludes that interference with glucose reabsorption could not be due to the inhibition of phosphatase.

Reviews of clinical interest on phosphatase have appeared (14, 15, 16).

Specific phosphomonoesterases. — Gomori (17) has obtained enzyme preparations from kidney and liver, by extraction in lactate buffer at pH 3.5, which have negligible activity for β-glycerophosphate or for phenylphosphate but hydrolyze commercial preparations of hexosediphosphate. Activity on hexosemonophosphate was not determined. The pH optimum is 9.7; the enzyme requires magnesium ions for activity; it is activated by cyanide and inhibited by fluoride. Muscle, extracted by this method, yields only small amounts of hexosediphosphatase although Lohmann showed that saline extracts of muscle can hydrolyze fructose-1,6-diphosphate rapidly to form hexose-6-phosphate.

An enzyme from snake venom is specific for adenosine-5-phosphate and inosine-5-phosphate, splitting them into nucleoside and inorganic phosphate (18). Enzymes occurring in Basidiomycetes split α- or β-glycerophosphate but not other mono- or diesters of phosphate (19).

Phosphodiesterase

The essential characteristic of a diesterase is that it splits only one of the ester linkages. The second ester linkage is split by phosphomonoesterase which liberates inorganic phosphate. The alkaline phosphatase of Schmidt & Thannhauser (4) splits both linkages of diphenylphosphate.

The phospholipids are diesters of phosphoric acid. Splitting of these ester linkages in lecithin must be preceded by the action of lecithinase which splits off the fatty acid molecules. There are two possible ways of breaking down the remainder of the molecule depending on

which phosphate ester linkage is split first, i.e., that with choline or that with glycerol. Phosphate is also hydrolyzed from the tubercle phosphatide by phosphatases after preliminary action by lecithinase (20).

Ribonucleic acid (yeast nucleic) and desoxyribonucleic acid (thymonucleic) are similar in structure since they are polymers of mononucleotides which are probably linked to each other by an ester linkage between the phosphate group of one nucleotide and the sugar molecule of the neighboring nucleotide. They are thus diesters of phosphoric acid. The depolymerizing agents of the macromolecules are ribonuclease and thymonucleodepolymerase. These enzymes are specific for their respective nucleic acids (24).

Pyrophosphatase

Adenosinetriphosphatase.—Whereas the hydrolysis of a simple phosphoric ester by phosphatase is accompanied by the liberation of a relatively small amount of free energy, and can be reversed to a demonstrable extent under certain conditions, the hydrolysis of the terminal pyrophosphate bond of ATP, with the formation of ADP and inorganic phosphate, is accompanied by the release of a large amount of free energy and is not measurably reversible. According to Lipmann's terminology (3), the pyrophosphate bonds of ATP are energy-rich, while the phosphoric ester bonds are energy-poor. The enzymatic hydrolysis of ATP by adenosinetriphosphatase is generally considered to be the immediate chemical source of energy for muscular contraction, and perhaps also for other types of cellular work (3, 28), including the fixation of carbon dioxide by plants (29) and autotrophic bacteria (30).

In 1939, Engelhardt & Lyubimova (31, 32) made the important observation, since confirmed by others (28, 33), that the adenosinetriphosphatase activity of muscle is almost entirely associated with myosin, the globulin which constitutes 57 to 70 per cent of the total protein of muscle tissue (34) and is believed to be responsible for the contractile and elastic properties of muscle. The evidence which has been submitted in favor of the viewpoint that adenosinetriphosphatase is actually identical with myosin may be summarized as follows: (*a*) The adenosinetriphosphatase activity of myosin remains fairly constant on reprecipitation by dialysis, dilution, acidification or salting out (31, 32, 34). (*b*) In no case has a protein fraction been obtained from myosin

having a significantly higher activity than the original material (31, 32, 34). (*c*) Certain mild procedures which are known to cause denaturation of myosin, as indicated by the loss of flow birefringence, cause complete loss of adenosinetriphosphatase activity (31, 32). (*d*) Myosin, precipitated three times by dilution, shows a single sharp ascending boundary during cataphoresis at pH 6.8 (34). (*e*) The solubility of myosin in a buffer of given ionic strength and pH is independent of the amount of myosin present in the solid phase (35, 36). (*f*) The adenosinetriphosphatase activity of myosin, expressed as moles of substrate transformed per unit weight of protein, is of the same order of magnitude as the activity of certain of the less active pure enzymes (34, 35). (*g*) Addition of small amounts of ATP to myosin sols or artificially prepared myosin threads causes certain changes, described below, in the properties of the myosin, indicating a reaction of ATP with myosin which is interpreted by some workers as an enzyme-substrate combination.

On the other hand, there is some evidence that the usual myosin preparation is not a single protein but is merely difficult to separate from small amounts of a highly active adenosinetriphosphatase: (*a*) There is no relationship between the degree of birefringence and the enzyme activity of myosin preparations from different species (34). (*b*) Flow birefringence can be abolished by certain procedures without decreasing adenosinetriphosphatase activity (34). (*c*) In the cataphoresis experiments described by Bailey, the descending boundary showed a second component moving 0.8 times as fast as the main boundary (34). (*d*) Myosin can be separated centrifugally into four proteins having different sedimentation constants (37). (*e*) A water-soluble adenosinetriphosphatase can be prepared from potatoes (38) which is 20 times as active as myosin per mg. protein (39). This enzyme is adsorbed by myosin when the latter is precipitated by dilution (39).

It should be emphasized that even if future work should prove that myosin and adenosinetriphosphatase are not identical, the close association of the two proteins might still be highly significant.

Investigations of the enzymatic properties of myosin indicate that it is a specific triphosphatase. Of the many phosphate compounds investigated (28, 34, 40) only ATP, inosinetriphosphate (ITP) (41, 42), and inorganic triphosphate (41) can be hydrolyzed. With purified myosin (reprecipitated several times), only the terminal phosphate is split from either ATP or ITP, so that ADP (40) and IDP

(42) can be isolated in pure form by this means. Although the rate of enzymatic hydrolysis of ITP is greater than that of ATP, it seems unlikely that ITP is the physiological energy source since the inosine nucleotide is not nearly as effective as the adenine nucleotide in various transphosphorylation reactions (42).

The adenosinetriphosphatase activity of myosin is increased markedly by the addition of calcium ions (33, 34, 36). The concentration of calcium required for optimal activity depends on the substrate concentration, 1 mole of calcium salt being required per 1.5 moles of ATP (34). A water-soluble adenosinetriphosphatase preparation which is not activated by calcium ions has been obtained from liver (34). However, the activity of crude dispersions, containing both the water-soluble and insoluble proteins of liver or other tissues, is increased several-fold by the addition of calcium ions (43).

The activating effect of calcium ions on myosin preparations has led Bailey (34) to propose that the process of muscular contraction is characterized by the liberation of calcium ions, which causes hydrolysis of ATP to take place. DuBois & Potter (44) claim that the adenosinetriphosphatase activity of homogenized salivary gland tissue, tested without the addition of calcium, is increased by preliminary incubation of the intact tissue with acetylcholine, and suggest that acetylcholine causes an increase in the amount of calcium available for activation of the enzyme. The effects of acetyl choline, epinephrine, and other substances on the enzyme activity of myosin have been tested in the presence of optimal amounts of added calcium ions with negative results (45).

The adenosinetriphosphatase activity of myosin is increased not only by calcium, but also to a lesser extent by manganese (34). Among other cations tested, barium and ferrous iron have no effect (34, 36), while copper (34) and silver (36) are strongly inhibitory. With purified myosin, magnesium has no effect according to Bailey (34), but Engelhardt & Lyubimova (36) find that it has a strong inhibitory effect. DuBois *et al.* (46) find a higher ATP content in the brain and muscle in magnesium-anesthetized animals than in ether-anesthetized animals, and suggest that the production of anesthesia by magnesium salts may be due to competition of the magnesium ions with calcium ions for adenosinetriphosphatase.

The pH optimum for the enzymatic activity of myosin is about 9.0 (31, 34). There is a second, less pronounced, optimum at pH 6.2 (36). The existence of two optima is attributed to the dependence of

activity on the ionization of both ATP and adenosinetriphosphatase, rather than to the presence of two different enzymes. The inactivation of the enzyme at 37° is prevented by the presence of ATP or ADP, especially the former (31). Both the stability and activity of the enzyme are increased by amino acid buffers, including cysteine (34). Barron & Singer (47) report that the enzyme contains sulfhydryl groups essential to its activity, since it is inactivated by chlormercuribenzoic acid and completely reactivated by glutathione. Other workers, using other reagents which react with sulfhydryl groups, such as iodoacetic acid, come to the opposite conclusion (28, 36). Cyanide has no effect on the activity (36). Fluoride completely abolishes the stimulatory effect of added calcium but has no effect on the activity in the absence of added calcium (36). Inhibition occurs with phlorhizin only at acid reaction (36), and with high potassium chloride concentrations (48).

Evidence that ATP may be directly responsible for changes in the mechanical properties of muscle has been presented by Engelhardt *et al.* (35, 36, 49) and by Needham *et al.* (41, 50). The Russian workers (35, 36, 49) find that when myosin threads, prepared by extrusion of a salt solution of myosin into water, are subjected to a given load, their extensibility is increased by the addition of low concentrations of ATP (0.0025 *M*). The phenomenon is specific for phosphate anhydrides, being observed to a smaller extent with ADP, thiamin pyrophosphate, and cozymase. The effect is immediate and can be reversed when the thread is returned to water or dilute salt solution. Conditions which abolish the enzyme activity of myosin also abolish the effect of ATP on the extensibility of the myosin fibril.

According to the English workers (41, 50) when a myosin sol is treated with a low concentration of ATP or ITP, a rapid reduction in flow birefringence and in relative viscosity occurs, with no change in anomalous viscosity. Other workers, however, could find no effect of ATP on the viscosity (36) or on the ultraviolet absorption spectrum (51) of myosin sols. The changes observed by Needham *et al.* are reversible: both birefringence and viscosity return to normal values concurrently with the hydrolysis of ATP to ADP. Inorganic triphosphate, although hydrolyzed by the myosin preparation, had no effect on its birefringence or viscosity. Other pyrophosphate compounds, including ADP and thiamin pyrophosphate, also had no effect. Although treatment with other substances, such as urea or certain cations, produced a fall in birefringence, the molar concentration re-

quired was several hundred times the optimum concentration of ATP. Furthermore, these substances do not produce a simultaneous fall in viscosity and there is no spontaneous reversal. However, when birefringence has been lowered by these substances it can be restored by precipitating and redissolving the protein. This may indicate that the reversibility observed with ATP is merely due to hydrolytic removal of the ATP rather than to recharging of the myosin fibrils by the energy released on ATP hydrolysis.

Although the English and Russian workers both agree, in their latest publications (36, 41), that the immediate effect of ATP on myosin is the result of an enzyme-substrate type of combination of ATP with myosin, and not of the hydrolysis of ATP or of the phosphorylation of myosin, their experimental results disagree inasmuch as the former believe that the combination results in a contraction, while the latter find that combination results in an extension of the myosin fibril.

The observed effects of ATP on myosin may possibly be related to changes in the potassium content of myosin, since Montigel (52) reports that treatment of crude myosin solutions with ATP, ADP, choline, or acetylcholine causes a marked decrease in the potassium content of the myosin obtained on subsequent dilution and washing with water.

Inorganic pyrophosphatase.—This enzyme, in contrast to adenosinetriphosphatase, is activated by magnesium ions and inhibited by calcium ions (34).

Diethyl or diphenyl pyrophosphatase.—These enzymes have a wide distribution. The ratio of the concentration of pyrophosphatase to glycerophosphatase is greater in certain plants than in animal sources (53). Basidiomycetes (19) contain two pyrophosphatases with pH optima at about 4 and 6, respectively.

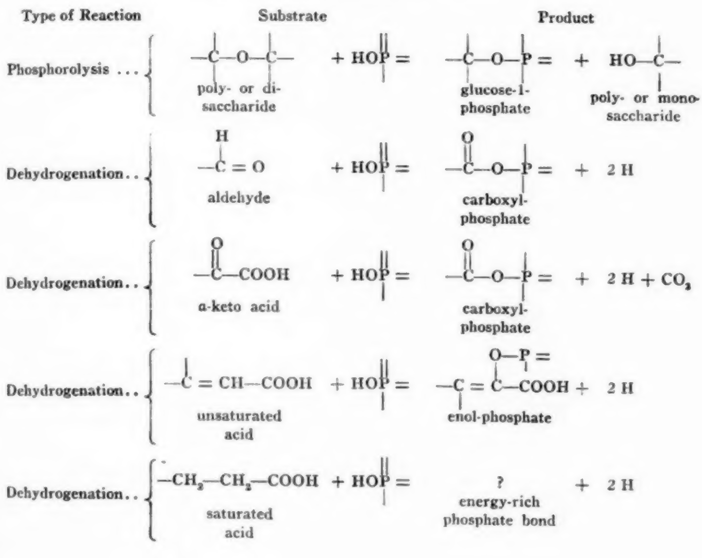
Cocarboxylase phosphatase. — The dephosphorylation of diphosphothiamin by "top yeast" phosphatase involves the rapid splitting of the pyrophosphate linkage followed by the slower splitting of the ester linkage of monophosphothiamin (54, 55). Both reactions are inhibited in the presence of thiamin (55). Thiamin also inhibits the splitting of glycerophosphate by top yeast phosphatase (56) but does not inhibit the splitting of either diphosphothiamin or glycerophosphate by animal tissue enzymes (56, 57). Cocarboxylase when combined with protein to form carboxylase is not so readily attacked by phosphatase as when in the free state (58, 59).

CREATION OF PHOSPHATE BONDS

The reactions discussed in the previous section are practically irreversible under physiological conditions. The reactions in the table below are those by which inorganic phosphate can be taken up by organic compounds. There are two main types, those catalyzed by phosphorylase, and the remainder in which phosphate esterification is associated with oxidation. They have this in common: in both types phosphoric acid plays a role commonly played by water, and a reaction

TABLE II

CREATION OF PHOSPHATE BONDS



which would otherwise be irreversible becomes reversible. The glucosidic linkages in a di- or polysaccharide are split irreversibly by a hydrolytic enzyme but are split reversibly by phosphorylase to form glucose-1-phosphate. Biological oxidation of certain substances containing double bonds can be interpreted in terms of the addition of water and the

irreversible removal of hydrogen, or as the addition of phosphoric acid and the reversible removal of hydrogen.

Phosphorylase

Polysaccharide phosphorylase.—This is the enzyme that catalyzes the first step in the degradation of polysaccharide (starch or glycogen) or, since the reaction is reversible, the last step in its synthesis, according to the reaction, polysaccharide + inorganic phosphate ⇄ glucose-1-phosphate. Thus a glucose unit is esterified with phosphate by a process of phosphorolysis.

The synthesis of dextran from sucrose (60) in bacteria, is analogous to the synthesis of polysaccharide from Cori ester. Maltosidic units are formed and fructose instead of inorganic phosphate is liberated. Similarly, an enzyme extracted from certain bacteria forms levan, a non-reducing polysaccharide consisting of fructose units, from sucrose, with the production of reducing sugar (61).

The detailed procedure for the preparation of the crystalline muscle phosphorylase has been reported (62). The molecular weight, determined by the ultracentrifuge, is between 340,000 and 400,000 (63). Cysteine increases the solubility and the activity of the crystalline enzyme. AMP also increases its activity but the enzyme shows about 65 per cent of its maximum activity without added AMP (64). Under optimal conditions, 4×10^4 molecules of glucose-1-phosphate are converted to polysaccharide per molecule of enzyme per minute at 30° (65). Although the ratio, inorganic phosphate to glucose-1-phosphate, at equilibrium varies considerably with pH, the ratio of the divalent ions of these two acids remains constant at a value of about 2 (65, 66). The inhibition of phosphorylase by glucose (65, 67), phlorhizin, or ammonium sulfate (65) is counteracted by small amounts of adenylic acid.

The crystalline muscle phosphorylase can be converted, by incubation with trypsin at pH 6, or by incubation with "PR" enzyme, to a more soluble form which is inactive without added AMP but is restored to its original activity in the presence of added AMP. The "PR" enzyme is a euglobulin present in muscle and spleen extracts (64).

Phosphorylase requires added glycogen for the synthesis of polysaccharide. This fact and the effect of varying concentrations of the added glycogen have led to the formulation of the theory (65) that the reaction is in reality glucose-1-phosphate + terminal glucose units ⇄ maltosidic chain units + inorganic phosphate. That is, the added

glycogen actually enters into the reaction, and "polysaccharide synthesis consists in a lengthening of the side chains of glycogen by addition of glucose units in 1 : 4 glucosidic linkage."

The polysaccharide formed by both potato phosphorylase (68, 69) and crystalline muscle phosphorylase (70) has been found to consist of long unbranched chains of glucopyranose units joined in 1 : 4 glucosidic linkages.

Crystalline phosphorylase acting with a supplementary enzyme obtained by Cori & Cori (71) from heart or liver produces a branched type of polysaccharide resembling glycogen. It is suggested that this enzyme may be another type of phosphorylase which can make 1 : 6 glucosidic linkages. According to Meyer (72) potato phosphorylase can break only 1 : 4 linkages, while yeast phosphorylase, which synthesizes a polysaccharide similar to glycogen, contains enzymes which act on both 1 : 4 and 1 : 6 linkages.

Relative amounts of phosphorylase in different tissues have been studied recently (67). Stimulation of muscle has no effect on the total concentration (64, 73) but decreases the yield of crystalline phosphorylase (64). Phosphorylase is present in adipose tissue (67, 74) as is also glycogen (74, 75, 76). Glycogen appears and disappears rapidly (75) depending on the nutritional state of the animal, being increased when fat synthesis from carbohydrate is taking place and after insulin injection (76).

Disaccharide phosphorylase. — Certain bacterial preparations can split sucrose by phosphorolysis into glucose-1-phosphate and fructose (77, 78). The resulting Cori ester has been isolated and characterized chemically. The reaction is reversible and only glucose-1-phosphate and fructose can be utilized. Inorganic phosphate is esterified in the presence of sucrose but not in the presence of glucose or of fructose. Doudoroff (79) has extracted the sucrose phosphorylase from dried bacteria and obtained it relatively free from invertase and phosphatase by ammonium sulfate precipitation. No coenzyme is needed, and glucose inhibits the phosphorolytic process. The equilibrium constant, K, which is equal to [sucrose] [inorganic phosphate]/[glucose-1-phosphate] [fructose], has a value of 0.05 at pH 6.6 and of 0.09 at pH 5.8 at 30°.

The synthesis of sucrose in sugar cane may also be mediated by a phosphorylase, since phosphatase inhibits the formation of sucrose, thus suggesting the necessity for participation of a phosphate ester (80).

Phosphate Esterification by Oxidation

The esterification by phosphorylase produces only low-energy phosphate bonds. High-energy phosphate bonds are formed from inorganic phosphate by phosphorylative oxidation. The essential feature of this process is the addition of inorganic orthophosphate to a double bond accompanied by dehydrogenation.

Aldehyde oxidation.—The oxidation of 3-phosphoglyceraldehyde involves, according to Warburg, the addition of inorganic phosphate to form 1,3-diphosphoglyceraldehyde, which is then oxidized to 1,3-diphosphoglyceric acid. Meyerhof & Junowicz-Kocholaty (81) have been unable to find any experimental evidence for the intermediary formation of 1,3-diphosphoglyceraldehyde in this oxidation, and therefore postulate the formation of a "loose physical addition product." Whether the addition be chemical or physical, the oxidation causes the incorporation of inorganic phosphate into carboxyl phosphate, which can then react with ADP to form ATP, so that the energy derived from the oxidation is conserved in the ATP. Such a synthesis of ATP from inorganic phosphate can occur in intact cells during glucose fermentation (82).

α-Keto acid oxidation.—Lipmann (83) showed that the oxidation of pyruvate by *L. delbrückii* requires, in addition to inorganic phosphate, a divalent cation, cocarboxylase and flavine adenine dinucleotide, and that the products of oxidation are acetyl-phosphate and carbon dioxide. Active cell-free extracts have been obtained from *E. coli* (84) and *Clostridium butylicum* (85) which catalyze a similar reaction but require an unidentified heat-stable factor.

Lipmann (3) has suggested that the oxidation product can function either as a phosphate donor or an acetate donor. There is indirect evidence that acetyl-phosphate can acetylate *p*-aminobenzoic acid *in vivo* (86) and choline (87, 88) in tissue extracts.

The general participation of inorganic phosphate in aerobic oxidations in animal tissues is indicated by the fact that its addition increases the oxygen consumption of a wide variety of tissues *in vitro* (89, 90). The incorporation of inorganic phosphate into a high-energy phosphate bond during such oxidation has been demonstrated by many workers, either by adding a large amount of AMP and showing ATP synthesis, or by adding a catalytic amount of adenine nucleotide and a large amount of a phosphate acceptor such as creatine, glucose, or

hexosemonophosphate. However, the direct demonstration in animal tissues of phosphorylated oxidation products similar to acetyl-phosphate has not yet been achieved.

The oxidation of pyruvate in tissue dispersions requires the presence of inorganic phosphate, and catalytic amounts of a divalent cation, thiamin pyrophosphate, a 4-carbon dicarboxylic acid, and adenine nucleotide (91). Potassium ion, although not stimulating the oxidation, has been found to increase the amount of phosphate esterification coupled with pyruvate oxidation (92). The stimulatory effect of inorganic phosphate and adenine nucleotide has also been observed with other α-keto acids, namely α-ketoglutarate (93) and α-ketobutyrate (94). Phosphate can be replaced by arsenate in the anaerobic dehydrogenation of pyruvate or α-ketobutyrate by brain dispersions (94) and in the aerobic oxidation of pyruvate by *L. delbrückii* (83), but not in the aerobic oxidation of pyruvate or α-ketobutyrate by brain dispersions (94), or of α-ketoglutarate by heart extract (93). The oxidation of α-ketoglutarate to succinate does not require the addition of a 4-carbon-dicarboxylic acid (93). This may indicate that the latter, although necessary for the complete dehydrogenation of pyruvate by way of the citric acid cycle, is not essential for the transport of the hydrogen to oxygen.

Ochoa finds that the one-step oxidation of α-ketoglutarate to succinate (93) can give the same amount of phosphate esterification per atom of oxygen as that observed in the complete oxidation of pyruvate (95) by heart extract, namely three atoms of phosphorus per atom of oxygen. Such a ratio cannot be accounted for by the mechanism described above by which the removal of a pair of hydrogen atoms from the substrate is accompanied by the esterification of only one molecule of phosphate. The subsequent transfer of hydrogen to oxygen would seem to bring about the esterification of two additional molecules of phosphate. The energy derived from the passage of two hydrogen atoms from the potential level of the α-keto acid to that of oxygen is equivalent to that required for the formation of five energy-rich phosphate bonds, so that the observed esterification corresponds to an efficiency of 60 per cent (95).

Succinic acid oxidation.—When succinate is used as substrate, its oxidation to fumarate causes the esterification of not more than one molecule of phosphate (95, 96). The lower ratio of phosphorus to oxygen in this case is to be expected, in view of the relatively high potential of the succinic-fumaric system. The mechanism

by which inorganic phosphate is taken up in this case remains to be elucidated.

Malic acid oxidation.—In the oxidation of malic acid to oxaloacetic one might expect, on the basis of the potential of this system, the esterification of two phosphate molecules per atom of oxygen. No exact measurements of this ratio are available, but it has been shown by Kalckar (2) and since confirmed (97) that malic acid oxidation can cause the uptake of inorganic phosphate with the formation of phosphopyruvate. The mechanism for this reaction as proposed by Lipmann (3) involves the addition of inorganic phosphate to fumarate followed by oxidation to phospho-(enol)-oxaloacetate (type *2c*) which could then be decarboxylated to phosphopyruvate.

Fatty acid oxidation.—The esterification of inorganic phosphate appears to be involved in the oxidation of saturated fatty acids. A dialyzed extract of adipose tissue has been found to catalyze the reduction of methylene blue by long chain fatty acids only in the presence of inorganic phosphate and AMP (98). No detectable amount of inorganic phosphate disappeared, as might be expected, since the complete reduction of the methylene blue could have caused the esterification of only 0.1 per cent of the phosphate present. Muñoz & Leloir (97) find that the aerobic oxidation of certain lower saturated fatty acids by an enzyme preparation from liver requires not only inorganic phosphate and adenine nucleotide, but also fumarate, cytochrome-*c*, and magnesium or manganese ions. When the fatty acid is omitted from this system, fumarate is oxidized, inorganic phosphate disappears, and phosphopyruvate accumulates, presumably according to the mechanism described above. However, when fatty acid is being oxidized, no inorganic phosphate disappears and no phosphopyruvate accumulates. The necessity for phosphate in the oxidation of fatty acids remains to be explained. As is the case with succinate, there are no double bonds at which an addition of phosphate might be expected.

According to the multiple alternate oxidation theory, when a C_{16} fatty acid is oxidized, four molecules of acetoacetic acid are formed. Recent work (99, 100) indicates that the latter may be oxidized, like pyruvate, via the citric acid cycle. If so, the energy derived from the complete combustion of fat, as well as carbohydrate, may be stored in energy-rich phosphate bonds.

Oxidative formation of inorganic pyrophosphate.—Another type of phosphate esterification coupled with oxidation occurs in liver dispersions (95, 101) and molds (102) in which inorganic phosphate

disappears and inorganic pyrophosphate has been isolated as the product. The pyrophosphate may have been formed from a labile organic compound during isolation.

PHOSPHATE TRANSFER

Phosphate transfer from one linkage to another can occur either intramolecularly or between two molecules. Whereas the latter always requires the intermediation of adenine nucleotides, the former can occur without them.

TABLE III

PHOSPHATE TRANSFER

	(a) Intermolecular Transfer of Phosphate
ATP formation	ADP + phosphopyruvate → ATP + pyruvate ADP + 1,3-diphosphoglycerate ⇌ ATP + 3-phosphoglycerate ADP + acetylphosphate → ATP + acetate
Storage of energy-rich phosphate bonds	ATP + creatine ⇌ ADP + phosphocreatine ATP + arginine ⇌ ADP + phosphoarginine
ATP utilization	ATP + glucose → ADP + glucose-6-phosphate ATP + fructose-6-phosphate → ADP + fructose-1,6-diphosphate
Internucleotide phosphate transfer	ADP + ADP ⇌ ATP + AMP
	(b) Intramolecular Transfer of Phosphate
	glucose-1-phosphate ⇌ glucose-6-phosphate 3-phosphoglycerate ⇌ 2-phosphoglycerate

INTERMOLECULAR TRANSFER OF PHOSPHATE

The adenine nucleotides have the ability to take part, as substrate, in a wide variety of enzyme-substrate combinations, so that ADP (or AMP) can accept phosphate from a wide variety of energy-rich phosphate compounds formed at the expense of metabolism. The energy-rich phosphate bond of the ATP so formed can be utilized: (*a*) by hydrolysis, or (*b*) to phosphorylate a large number of compounds; or it can be stored by transfer of the terminal phosphate to creatine or arginine. It is of some interest that the adenylthiomethylpentose of yeast, which Lipmann (3) has suggested may play an analogous role in methyl transfer, appears to have exactly the same configuration as muscle adenylic acid, but with a thiomethyl instead of a phosphate group on the C_5 of *d*-ribose (103).

In addition to the well-known adenine-9-*d*-ribose-5′-phosphoric acid mononucleotides (AMP, ADP, ATP) from muscle, there are

other nucleotides which can play a similar role in the transport of phosphate. These include the di-(adenosine-5′-phosphoric acid) nucleotide isolated from yeast by Kiessling & Meyerhof (104) and a new coenzyme isolated from yeast by Ohlmeyer (105, 106) but not yet completely identified. The new coenzyme is reported in fact to be much more effective than the nucleotides from muscle in catalyzing the fermentation of hexosemonophosphate by either yeast or muscle extracts. However, its greater effectiveness in this system is difficult to explain, since it is no more effective than the known mononucleotides in any of the transphosphorylation reactions studied, including the transfer of phosphate from phosphopyruvate to hexosemonophosphate or creatine. Like ADP and di-adenosinetetraphosphate, the new coenzyme contains 50 per cent of its phosphate in acid-labile form and shows the typical ultra-violet absorption maximum at 260 mμ, but differs in that it has a low amino-nitrogen content. Its silver salt is less soluble than that of the known monoadeninenucleotides and its stability is reported to be greater than that of the known diadeninenucleotides. Unlike ADP, but like diadenosinetetraphosphate, it cannot be further phosphorylated by phosphopyruvic acid.

ATP formation.—Although a large number of "energy-rich" phosphate compounds, capable of phosphorylating ADP or AMP, are probably formed at the direct expense of metabolism, only diphosphoglycerate, acetylphosphate, and phosphopyruvate have been isolated so far.

Boyer *et al.* (92, 107) have presented indirect evidence that potassium ions (or ammonium ions) specifically stimulate while calcium ions and oxalate inhibit the reaction of phosphopyruvate with AMP or ADP. They report that magnesium or manganese ions in addition to potassium are essential for this transfer. In a direct study of the reaction of phosphopyruvate with ADP a marked stimulation by potassium ions was found (38). The reaction with ADP proceeds much more rapidly than that with AMP (92, 107). In cell-free preparations of *E. coli,* the reaction of phosphopyruvate with AMP was not affected by potassium but markedly stimulated by magnesium or manganese ions and inhibited by fluoride (108).

The dephosphorylation of phosphopyruvate usually proceeds by way of the above reaction with adenine nucleotide followed by adenosinetriphosphatase action. The liberation of inorganic phosphate from phosphopyruvate (or phosphoglycerate) in the absence of adenosinetriphosphatase, but in the presence of catalytic amounts of adenine

nucleotide, is due to the formation of 1,3-diphosphoglycerate which undergoes non-enzymatic hydrolysis to some extent during incubation (109). This compound, if not hydrolyzed during incubation, would certainly undergo complete hydrolysis during the inorganic phosphate estimation. This type of dephosphorylation probably has no physiological importance (109). The acceleration of this dephosphorylation by arsenate is due to the fact that the reduction of 1,3-diphosphoglycerate to yield 3-phosphoglyceraldehyde and inorganic phosphate, is not counterbalanced by the oxidation of 3-phosphoglyceraldehyde, which proceeds in the presence of arsenate without uptake of inorganic phosphate (109).

Storage of energy-rich phosphate bonds.—Phosphocreatine (in vertebrates) is usually regarded as the storehouse for energy-rich phosphate bonds, derived from ATP according to the reaction, ATP (ADP) + creatine ⇄ phosphocreatine + ADP (AMP). When ATP is being utilized, the reaction can proceed in the opposite direction.

Evidence has been presented that phosphocreatine can serve as the source of energy for the discharge of electrical impulses in the electric organ of *Electrophorus electricus* (111). It is suggested that the energy of phosphate bonds is used for the resynthesis of acetylcholine (87).

The rapid rate at which the phosphate of ATP and phosphocreatine is renewed from intracellular inorganic phosphate during resting metabolism is well illustrated by the experiments of Furchgott & Shorr (112). Cardiac muscle slices, after respiring at 37°, in a medium containing radioactive inorganic phosphate, were washed free of the highly radioactive extracellular phosphate. The remaining intracellular inorganic phosphate showed a much lower radioactivity, corresponding exactly to that of the terminal phosphate of ATP and the phosphate of phosphocreatine. The other labile phosphate group of ATP was relatively low in radioactivity, as might be predicted from its lower reactivity in enzymatic reactions. In view of the low rate of exchange between intracellular and extracellular inorganic phosphate in comparison with the rapid rate of incorporation of intracellular inorganic phosphate into ATP and phosphocreatine, it is not surprising that Sacks (113) and Flock & Bollman (114) failed to find an increase in the radioactivity of the organic phosphate fractions of muscle upon stimulation. It is generally agreed that the rate of exchange between extra- and intracellular inorganic phosphate is not increased during work (114, 115). Furchgott & Shorr (112) found

that at 2° metabolism was sufficiently slowed so that the intracellular inorganic phosphate showed a higher radioactivity than the organic phosphate fractions. This indicates that inorganic phosphate can enter the cell directly without the mediation of the organic phosphate carrier postulated by Sacks (116).

Utilization of ATP.—The utilization of the pyrophosphate bond energy of ATP for the formation of energy-poor phosphate bonds, as in the formation of fructose-1,6-diphosphate from fructose-6-phosphate, is an irreversible reaction accompanied by the release of a large amount of free energy.

In addition to glucose and fructose-6-phosphate, a wide variety of other compounds containing alcoholic hydroxyl groups have been shown to accept phosphate from ATP. These include various free hexoses, glycerol, adenosine (96), gluconic acid (117), thiamin (54), and diphosphopyridine nucleotide. Although it has been reported that oxidation can cause an increased phosphorylation of glycogen (118), glycogen undoubtedly does not accept phosphate from ATP, but undergoes phosphorolysis, ultimately forming fructose-6-phosphate, which can then act as phosphate acceptor.

Formation of hexosemonophosphate. — The enzyme hexokinase which catalyzes the transfer of phosphate from ATP to hexoses is probably present in all cells which can ferment glucose. Huszák (119) reports that the hexokinase of brain is present in the gray, but not in the white matter. Hexokinase from yeast catalyzes the reaction, ATP + glucose → ADP + glucose-6-phosphate. ADP can be isolated in this way, since it can not act as phosphate donor to glucose in this system (120). The possibility of a primary formation of glucose-1-phosphate, followed by conversion to glucose-6-phosphate has been ruled out (120). Fructose is also phosphorylated at carbon atom 6 by yeast hexokinase (121), but there may be an enzyme in liver which can cause its phosphorylation in position 1, since when fructose is added to respiring liver dispersions, fructose-1-phosphate can be isolated in large amounts from the phosphoric esters which accumulate in the presence of fluoride (121). Fructose-1-phosphate has also been isolated from the esters which accumulate in liver dispersions incubated without addition of fructose (122). Kosterlitz (123) reports that about 25 per cent of the mixture of esters isolated from the liver of rabbits fed galactose can be identified as galactose-1-phosphate by comparison with the properties of the synthetic ester. Galactose administration also appeared to cause a slight increase in the glucose-

1-phosphate content of the liver. Since the rates of fermentation of glucose-1-phosphate and galactose-1-phosphate by extracts of galactose-adapted yeast were found to be identical, while those of glucose and galactose differed, the author proposes that an equilibrium, galactose-1-phosphate $\rightleftarrows$ glucose-1-phosphate, may exist (124). Such a scheme could explain the formation of glycogen from galactose in the liver, as well as the formation of fructosediphosphate during fermentation of galactose in yeast extract.

The phosphorylation of glucose in respiring kidney dispersions containing inorganic phosphate and AMP is reported by Beck (13), in confirmation of Kalckar, to be inhibited by concentrations of phlorhizin low enough to satisfy Lundsgaard's hypothesis that phlorhizin prevents glucose reabsorption by inhibiting its phosphorylation. It remains to be determined whether it was the transfer of phosphate from ATP to glucose (hexokinase) or the oxidative synthesis of ATP which was inhibited by phlorhizin in these experiments.

The same question arises in the experiments of Kaplan & Greenberg (125), who found that the administration of glucose to rats given trace doses of radioactive phosphate caused an increase in the amount of P^{32} in the ATP of the liver in normal animals, but not in animals poisoned with phlorhizin, malonate, or fluoride. Administration of insulin alone also increased the amount of P^{32} in the ATP, the greatest increase being found when both glucose and insulin were administered. The increase in the amount of P^{32} in the ATP and inorganic phosphate fractions was due, at least in part, to actual increases in the amounts of these fractions after insulin administration (126). Sacks (127) reports that glucose administration to cats given radioactive inorganic phosphate did not increase the radioactivity of the ATP or phosphocreatine fractions of muscle but that insulin plus glucose caused a marked increase in the turnover of both organic phosphate fractions.

Formation of fructosediphosphate. — Fructose-1,6-diphosphate, which accumulates during glucose fermentation by yeast maceration juice, can be shown to be formed by the reaction of ATP with fructose-6-phosphate. Nilsson (128) and Enders & Sigurdsson (129), who consider fructosediphosphate "unfermentable" and regard its accumulation in extracts as an artifact, propose other mechanisms for its formation. However, since maceration juice lacks adenosinetriphosphatase, the accumulation of fructosediphosphate may readily be explained as an increase in the amount of ATP reacting with hexosemonophosphate (1), without resorting to unproved hypotheses.

The enzyme catalyzing the reaction between ATP and fructose-6-phosphate is reported to be strongly inhibited by oxidizing agents. This inhibition is believed by Engelhardt & Sakov (130) to account for the Pasteur effect in yeast by blocking the fermentation pathway and allowing the oxidation of hexosemonophosphate by way of phosphogluconic acid (117). Evidence that the divergence between oxidative and anaerobic breakdown of carbohydrate may occur at the hexosemonophosphate rather than at the pyruvate stage in animal tissues is presented by Tankó (131) who finds carbon dioxide production aerobically from glycogen but not from fructosediphosphate in muscle dispersions.

The rate of formation of fructosediphosphate from ATP and hexosemonophosphate is reported to be remarkably stimulated by 1,2,4- or 1,2,5- but not by 1,2,6-dinitrophenol in a concentration of $10^{-3}M$ (132). This conclusion is based on the fact that the rate of carbon dioxide production and phosphate esterification during the inductive phase of fermentation of glycogen, glucose, or hexosemonophosphate in Lebedew juice is stimulated, while fermentation of fructosediphosphate is not influenced.

Myokinase.—Studies of the various enzymatic reactions by which phosphate is transferred to and removed from the adenine nucleotides reveal that the terminal phosphate of ATP is much more reactive than that of ADP; e.g., only the terminal phosphate of ATP can be split off by myosin or transferred to glucose by hexokinase, and, in the reverse direction, ADP is much more readily phosphorylated by phosphopyruvate or diphosphoglycerate than is AMP. Myokinase, a water-soluble enzyme found most abundantly in muscle, makes both labile phosphate groups equally reactive by catalyzing the reaction, 2 ADP $\rightleftarrows$ ATP + AMP (133). Myokinase appears to be specific for adenine nucleotides, since it has no effect on IDP (42). Although first detected by its ability to bring about the transfer of the terminal phosphate group of ADP to glucose in the presence of hexokinase (120) and by its ability to effect the hydrolysis of ADP by myosin (134), it is conceivable that myokinase might also increase the rate of phosphorylation of AMP by phosphopyruvate or diphosphoglycerate.

Myokinase is extremely stable, retaining 80 per cent of its activity after boiling for 10 minutes in 0.1 N hydrochloric acid, and appears to contain essential sulfhydryl groups (120). The enzyme, when partially purified, requires magnesium ion for optimal activity, and when

acting on ADP, can catalyze the transfer of about 4 μg. phosphorus per μg. protein per minute at 30° and optimal pH, 7.5; this corresponds to a turnover of more than 10,000 moles of phosphate per 100,000 grams of protein per minute. At equilibrium, about two-thirds of the added ADP has been converted. The same position of equilibrium appears to be reached when equimolar amounts of ATP and AMP are used as substrate (133).

Intramolecular Transfer of Phosphate

Phosphoglucomutase.—This enzyme, which catalyzes the transformation of glucose-1-phosphate to glucose-6-phosphate has been obtained free from other enzymes acting on these two substances (135). Pure glucose-6-phosphate can be prepared from glucose-1-phosphate with the enzyme, since the equilibrium mixture contains 95.5 per cent glucose-6-phosphate at 30°.

The rate of esterification of inorganic phosphate with glycogen in various types of muscle depends on the rate of removal of glucose-1-phosphate by phosphoglucomutase (73) so that measurement of the rate of inorganic phosphate disappearance is not a true measure of phosphorylase activity. The low rate of glycogen phosphorylation in certain tissues is due to low phosphoglucomutase activity (67, 74).

According to Montigel & Verzar, the rate of esterification of inorganic phosphate in fluoride-poisoned, glycogen-supplemented muscle brei from adrenalectomized animals is much lower than with normal animals but can be increased to normal by addition *in vitro* of desoxycorticosterone (136). This interesting observation, if confirmed, would merit further work to determine which enzyme in the crude muscle brei was actually affected by desoxycorticosterone. Muscle from adrenalectomized animals maintained with desoxycorticosterone (136) or salt (67) showed the normal rate of inorganic phosphate esterification.

Phosphoglyceromutase.—Whereas phosphoglucomutase activity is increased by divalent ions, phosphoglyceromutase appears to be inhibited by magnesium ions (108). The ratio of 3-phosphoglyceric acid to 2-phosphoglyceric acid at equilibrium at 20° has been calculated by Warburg (137) to be 2.67. This value is much lower than the values reported by Utter & Werkman (108) and Meyerhof & Schulz (138).

REACTIONS INVOLVING NO CHANGE IN POSITION OF PHOSPHATE

The reactions considered in this section and outlined in the table are limited to certain reversible reactions involved in carbohydrate fermentation, in which the phosphate group remains attached to the same carbon atom. No mention is made of many more such reactions of known biological importance, especially reactions involving phosphorylated derivatives of thiamin, riboflavin, nicotinic acid, and according to more recent work, α-tocopherol (139).

TABLE IV

REACTIONS INVOLVING NO CHANGE IN POSITION OF PHOSPHATE

Type of Reaction	Specific Reaction
Reversible aldol condensation....	fructose-1,6-diphosphate ⇌ glyceraldehyde phosphate + dihydroxyacetone phosphate
Equilibrium between keto-aldo isomers	glucose-6-phosphate ⇌ fructose-6-phosphate glyceraldehyde phosphate ⇌ dihydroxyacetone phosphate
Dehydration	2-phosphoglycerate ⇌ phospho-(enol)pyruvate + H_2O

Aldolase.—This is the enzyme which splits fructose-1,6-diphosphate into two triosephosphate molecules. The equilibrium constant for the aldolase reaction at various temperatures has been redetermined (81).

Warburg & Christian (140) have crystallized aldolase from a water extract of rat muscle, fractionating first with acetone and then with ammonium sulfate. At 38° and pH 7.4, 100,000 gm. of enzyme catalyze the breakdown of 7000 moles of hexosediphosphate per minute. In 1940, Herbert *et al.* (141) purified aldolase by fractional ammonium sulfate precipitation of a water extract of rabbit muscle and found that, at 38° and pH 7.3, 100,000 gm. of enzyme would split 3300 moles of hexosediphosphate per minute. Neither enzyme preparation contained metal ions. Engelhardt (35) reported that crystalline myogen (prepared according to Baranowski) showed aldolase activity which was retained after recrystallization for as many as four times. The activity reported is only a fraction of that of the Warburg crystals.

The activity of the crystalline enzyme of Warburg is not affected by complex formers, such as α-α′-dipyridyl, pyrophosphate, or cysteine. A partially purified enzyme from yeast (140) is inhibited by the above

complex formers. According to Warburg, enzymes so inhibited are dissociable compounds of heavy metals and the metal can be split off if the concentration of the free metal salts in the solution becomes sufficiently low. The inactivation by cysteine can be counteracted by the addition of divalent zinc, iron, cobalt, or copper ions, which combine with the cysteine. This combination is an equilibrium reaction governing the amount of free metal ion, so that the actual concentration of free metal ion bringing about reactivation is extremely low (of the order of $10^{-7}M$ for Zn). Reactivation by ferrous and cobaltous ions occurs only in nitrogen, since these ions are converted to the trivalent form in the presence of oxygen.

Triosephosphate isomerase. — Meyerhof & Junowicz-Kocholaty (81) have devised a new method for measuring small amounts of glyceraldehyde phosphate and have determined directly the equilibrium constant for the isomerase reaction (dihydroxyacetone phosphate)/(glyceraldehyde phosphate); they find it to be 20 to 25. This same value was obtained with either purified isomerase, or crude preparations containing aldolase (81), and also in determinations with ground *E. coli* (143).

Enolase. — Enolase catalyzes the formation of 2-phospho-(enol) pyruvate, containing an "energy-rich" phosphate bond, by the removal of water from 2-phosphoglycerate. The details of the method of crystallization of the enzyme as the inactive mercury salt have now been reported by Warburg & Christian (137). The yield is about 1 gm. of crystalline enzyme per kilo of dried brewers' yeast.

When freed of mercury, and combined with magnesium, manganese or zinc ions, the enzyme becomes active. The dissociation constant of the magnesium-enolase compound is $2.8 \times 10^{-3}M$ at pH 6.74 and $6.1 \times 10^{-4}M$ at pH 7.34. When fully saturated with magnesium, 50,000 gm. of protein are combined with 1 gm. mole of magnesium.

The enzyme is half-saturated with substrate by $1.5 \times 10^{-4}M$ 2-phosphoglycerate. When saturated, 10^5 gm. of enzyme convert 9900 moles of substrate per minute at 20° and pH 7.34. At equilibrium, the ratio of phosphopyruvate to 2-phosphoglycerate is 1.43. This value is lower than that found by Utter & Werkman (108) and Meyerhof & Schulz (138).

Massart & Dufait (11) and Utter & Werkman (108) reported independently of Warburg that enolase activity was increased by magnesium and manganese ions and attributed the fact that the magnesium enzyme was more strongly inhibited by fluoride than the manganese

enzyme to a greater stability of the magnesium-fluoride complex. These findings, as well as the observation of Meyerhof & Schulz (138) that the inhibition of enolase by fluoride is much decreased when inorganic phosphate is removed, are now explained by the following relationship between inhibition and the concentrations of phosphate, magnesium, and fluoride (137):

$$C_{Mg} \times C^2_{fluoride} \times C_{phosphate} \times \frac{\text{residual action}}{\text{inhibited action}} = K$$

$$K = 3.2 \times 10^{-12} \text{ at pH } 7.34 \text{ and } 20°.$$

The inhibition is due to the reversible replacement reaction: Magnesium fluorophosphate + magnesium enolase (active) $\rightleftarrows$ Magnesium fluorophosphoenolase (inactive) + magnesium salt. Arsenate, but not pyrophosphate, can replace phosphate in these reactions. The inhibition of carboxylase by fluoride involves a different mechanism, since it is not influenced by phosphate concentration (137).

Extracts of *E. coli* or of mammalian tissues capable of dehydrating phosphoglyceric acid are also able to dehydrate serine and remove hydrogen sulfide from cysteine in an analogous manner. All three types of activity are lost on dialysis and restored by the addition of zinc, magnesium, or manganese ions. Fluoride inhibits the action of the dialyzed reactivated extracts on cysteine and serine as well as on phosphoglycerate. Action of the extract on any of the above substrates could be inhibited by addition of either of the others (144).

DETERMINATION, PREPARATION, AND PROPERTIES OF PHOSPHATE COMPOUNDS

Methods of analysis.—10^{-3} to 10^{-4} μg. of inorganic phosphate can be determined by an ultramicro modification of the Fiske & Subbarow method (145). The true inorganic phosphate content of the blood has been reported to be much lower than that usually found; 65 to 70 per cent of the so-called inorganic phosphate results from the hydrolysis of phosphocreatine (146). Determination of inorganic pyrophosphate as the cadmium (148) and manganese (149) salts has been described.

α-Glycerophosphate has been determined in liver and other tissues by periodate oxidation and dephosphorylation of the diosephosphate by acid hydrolysis (150) or by alkaline peroxide at room temperature (151). β-Glycerophosphate can be determined by transposition to the

α-form in strong acid (150, 152). In mixtures of the two forms the relative amounts of each can be determined by the rate of hydrolysis at a pH greater than 3, where transposition does not take place (152). Neuberg & Lustig (153) report that the colorimetric method for the determination of phosphoglyceric acid with naphthoresorcinol is unreliable in the case of some biological material.

Kosterlitz (154) has determined the relative amounts of galactose-1-phosphate and glucose-1-phosphate on the basis of the more rapid rate of hydrolysis of the former in acid; the method has been applied to liver. Identification of glucose-6-phosphate as the osazone and fructose-1,6-diphosphate as the phenylhydrazone has been described (155).

A modification of the Bial reaction for determining pentose in which the ferric ion is replaced by cupric ion has been applied to the estimation of nucleotides (156). A micromethod for the determination of the total pentose content of tissues, including acid-insoluble pentose nucleoproteins, involves colorimetric determination of furfural obtained by distillation (157).

A spectrophotometric method for determination of triphosphopyridine nucleotide enzymatically has been described (158). Phospholipids have been determined by choline estimation (159) and by electrometric titration (160).

Occurrence of phosphorus compounds in tissues.—The phosphorylated carbohydrate esters of the autotrophic bacterium, *Thiobacillus thiooxidans,* appear to be identical with those of yeast and muscle (161), except for the ATP, which is adenosine-3-triphosphate (162). Other bacteria have been found to contain adenosine-5-triphosphate (162).

The fractionation of the acid-soluble phosphorus compounds of cerebral tissue frozen *in situ* showed the presence of ATP (163, 164), phosphocreatine, and compounds tentatively identified as hexose-6-phosphate and aminoethylphosphate (163). ATP and phosphocreatine are destroyed rapidly post mortem (163, 164). The content of phosphoglycerol and easily hydrolyzable phosphate of rat liver was decreased and inorganic phosphate increased on fasting (165). These changes could be reversed by feeding glucose (165). ATP has been isolated from liver and identified with that of muscle (166, 167). Fractionation of shock-producing muscle preparations resulted in the isolation of ATP which is probably responsible for the observed effects (168). Further evidence that adenine nucleotides are liberated from damaged cells has been reported (169, 170). Riboflavin phosphate is

suggested as the prosthetic group of purified acetoacetic decarboxylase (171). The coenzyme for *l*-lysine decarboxylase contains phosphorus (172) as does also the liver preparation active in pernicious anemia (173).

Chemical synthesis.—Glyceraldehyde 1,3-diphosphate (dimeric), synthesized by phosphorylation of dimeric *dl*-glyceraldehyde, is unable to replace glyceraldehyde-3-phosphate in various enzymatic reactions unless it has been subjected to partial hydrolysis in acid (174). An improved method for the synthesis of glyceraldehyde-3-phosphate has been reported by the same authors (175). *d*(—)-3-Phosphoglyceric acid and *d*(+)-2-phosphoglyceric acid have been synthesized and shown to be identical with the natural products (176). *d*-Fructose-6-phosphate has been prepared from *d*-fructose-1,6-diphosphate and the chemical properties of various salts of both substances described (177).

The phosphoric and pyrophosphoric esters of thiocholesterol and cholesterol (178) and the capric and lauric esters of glycerophosphate (179) have been synthesized. Thiamin orthophosphate has been synthesized by means of pure pyrophosphoric acid, and thiamin pyrophosphate by means of metaphosphoric acid (180). Adenosine-3-phosphate has been synthesized and shown to be identical with yeast adenylic acid (181).

Biological preparation.—A method for the precipitation of adenine nucleotides with aluminum picrate reagent and its application to the isolation of adenine nucleotides from yeast nucleic acid and beef heart has been described (182).

Fructose 1,6-diphosphate (183) and d(—)-3-phosphoglycerate (184) have been prepared by means of bakers' yeast and the same compounds together with hexosemonophosphate have been prepared from brewers' yeast from one fermentation mixture (185).

Physicochemical studies.—Physicochemical evidence for the incomplete dissociation of acid magnesium phosphate in solutions is presented (186). The pK_1' and pK_2' values of galactose-1-phosphoric acid are 1.00 and 6.17 (187). The acid strength of mono- and diesters of phosphoric acid has been investigated and interpreted in terms of solvation, and inductive, statistical and steric effects. The relationship of these effects to the reactivity of biologically important phosphate esters is discussed (188).

LITERATURE CITED[1]

1. Kalckar, H. M., *Chem. Revs.*, **28**, 71–178 (1941)
2. Kalckar, H. M., *Biol. Revs. Cambridge Phil. Soc.*, **17**, 28–45 (1942)
3. Lipmann, F., *Advances in Enzymology*, **1**, 100–62 (1941)
4. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369–85 (1943)
5. Sizer, I. W., *Proc. Soc. Exptl. Biol. Med.*, **49**, 700–3 (1942)
6. Albers, H., and Albers, E., *Z. physiol. Chem.*, **232**, 165–88, 189–95 (1935)
7. Caputo, R., and Marsal, A., *Rev. soc. argentina biol.*, **17**, 139–46 (1941)
8. Perlmann, G. E., and Ferry, R. M., *J. Biol. Chem.*, **142**, 513–17 (1942)
9. Cloetens, R., *Biochem. Z.*, **307**, 352–65 (1941); **308**, 37–39 (1941); **310**, 42–53 (1941)
10. Lawrie, N. R., *Biochem. J.*, **37**, 311–12 (1943)
11. Massart, L., and Dufait, R., *Z. physiol. Chem.*, **272**, 157–70 (1942)
*12. Kutscher, W., and Wüst, H., *Biochem. Z.*, **310**, 292–301 (1942)
13. Beck, L. V., *Proc. Soc. Exptl. Biol. Med.*, **49**, 435–39 (1942)
14. Sunderman, F. W., *Am. J. Clin. Path.*, **12**, 404–11 (1942)
15. Jaffe, H. L., and Bodansky, A., *Bull. N.Y. Acad. Med.*, **19**, 831–48 (1943)
16. Greenstein, J. P., *Advances in Enzymology*, **3**, 315–48 (1943)
17. Gomori, G., *J. Biol. Chem.*, **148**, 139–49 (1943)
18. Gulland, J. M., and Jackson, E. M., *Biochem. J.*, **32**, 597–601 (1938)
19. van Thoai, N., *Compt. rend.*, **214**, 643–44 (1942)
20. Gerstl, B., and Tennant, R., *Am. Rev. Tuberc.*, **46**, 600–611 (1942)
21. Bredereck, H., and Hoepfner, E., *Ber. deut. chem. Ges.*, **75B**, 1086–95 (1942)
22. Bredereck, H., and Jochmann, I., *Ber. deut. chem. Ges.*, **75B**, 395–400 (1942)
23. Fischer, F. G., and Lehmann-Echternacht, H., *Z. physiol. Chem.*, **278**, 143–54 (1943)
24. Greenstein, J. P., and Jenrette, W. V., *J. Natl. Cancer Inst.*, **2**, 301–3 (1941)
25. Loring, H. S., and Carpenter, F. H., *J. Biol. Chem.*, **150**, 381–88 (1943)
26. Rapoport, S., Leva, E., and Guest, G. M., *J. Cellular Comp. Physiol.*, **19**, 103–8 (1942)
27. Bolomey, R. A., and Allen, F. W., *J. Biol. Chem.*, **144**, 113–19 (1942)
28. Needham, D. M., *Biochem. J.*, **36**, 113–20 (1942)
29. Ruben, S., *J. Am. Chem. Soc.*, **65**, 279–82 (1943)
30. Vogler, K. G., and Umbreit, W. W., *J. Gen. Physiol.*, **26**, 157–67 (1942)
31. Lyubimova, M. N., and Engelhardt, W. A., *Biochimia*, **4**, 716–35 (1939)
32. Engelhardt, W. A., and Lyubimova, M. N., *Nature*, **144**, 668 (1939)
33. Szent-Györgyi, A., and Banga, I., *Science*, **93**, 158 (1941)
34. Bailey, K., *Biochem. J.*, **36**, 121–39 (1942)
35. Engelhardt, W. A., *Yale J. Biol. Med.*, **15**, 21–38 (1942)
36. Engelhardt, W. A., and Lyubimova, M. N., *Biochimia*, **7**, 205–30 (1942)
*37. Schramm, G., and Weber, H. H., *Kolloid-Z.*, **100**, 242–47 (1942)

[1] The articles marked with an asterisk were unavailable and only abstracts were consulted.

38. COLOWICK, S. P., SLEIN, M., AND BERGER, L. (Unpublished data)
39. KALCKAR, H. M., *J. Biol. Chem.* (In press)
40. LYUBIMOVA, M. N., AND PEVSNER, D., *Biochimia,* **6,** 178–83 (1941)
41. NEEDHAM, J., KLEINZELLER, A., MIALL, M., DAINTY, M., NEEDHAM, D. M., AND LAWRENCE, A. S. C., *Nature,* **150,** 46–49 (1942)
42. KLEINZELLER, A., *Biochem. J.,* **36,** 729–36 (1942)
43. DUBOIS, K. P., AND POTTER, V. R., *J. Biol. Chem.,* **150,** 185–95 (1943)
44. DUBOIS, K. P., AND POTTER, V. R., *J. Biol. Chem.,* **148,** 451–52 (1943)
45. ZIFF, M., *Proc. Soc. Exptl. Biol. Med.,* **51,** 249–51 (1942)
46. DUBOIS, K. P., ALBAUM, H. G., AND POTTER, V. R., *J. Biol. Chem.,* **147,** 699–704 (1943)
47. BARRON, E. S. G., AND SINGER, T. P., *Science,* **97,** 356–68 (1943)
48. MEHL, J. W., AND SEXTON, E. L., *Proc. Soc. Exptl. Biol. Med.,* **52,** 38–40 (1943)
49. ENGELHARDT, W. A., LYUBIMOVA, M. N., AND MEITINA, R. A., *Compt. Rend. Acad. Sci. U.R.S.S.,* **30,** 644–46 (1941)
50. NEEDHAM, J., SHEN, S.-C., NEEDHAM, D. M., AND LAWRENCE, A. S. C., *Nature,* **147,** 766–68 (1941)
51. LYUBIMOVA, M. N., AND SHIPALOV, M. S., *Biochimia,* **5,** 144–49 (1940)
52. MONTIGEL, C., *Helv. Physiol. Pharmacol. Acta,* **1,** C47–48 (1943)
*53. GIRI, K. V., *Ann. Biochem. Exptl. Med.,* **1,** 297–306 (1941)
54. OCHOA, S., "Cocarboxylase," *The biological action of the vitamins,* 17–42 (University of Chicago Press, 1942)
55. WESTENBRINK, H. G. K., VAN DORP, D. A., GRUBER, M., AND VELDMAN, H., *Enzymologia,* **9,** 73–89 (1940)
56. WESTENBRINK, H. G. K., AND VAN DORP, D. A., *Enzymologia,* **10,** 212–15 (1942)
57. GREIG, M. E., AND GOVIER, W. M., *J. Pharmacol.,* **79,** 246–49 (1943)
58. ENGELHARDT, W. A., AND WENKSTERN, T. W., *Biochimia,* **8,** 97–107 (1943)
59. WESTENBRINK, H. G. K., WILLEBRANDS, A. F., AND KAMMINGA, C. E., *Enzymologia,* **9,** 228–35 (1940)
60. HEHRE, E. J., *Proc. Soc. Exptl. Biol. Med.,* **54,** 240–41 (1943)
61. HESTRIN, S., AVINERI-SHAPIRO, S., AND ASCHNER, M., *Biochem. J.,* **37,** 450–56 (1943)
62. GREEN, A. A., AND CORI, G. T., *J. Biol. Chem.,* **151,** 21–29 (1943)
63. ONCLEY, J. L., *J. Biol. Chem.,* **151,** 27–28 (1943)
64. CORI, G. T., AND GREEN, A. A., *J. Biol. Chem.,* **151,** 31–38 (1943)
65. CORI, C. F., CORI, G. T., AND GREEN, A. A., *J. Biol. Chem.,* **151,** 39–55 (1943)
66. HANES, C. S., AND MASKELL, E. J., *Biochem. J.,* **36,** 76–79 (1942)
67. SHAPIRO, B., AND WERTHEIMER, E., *Biochem. J.,* **37,** 397–403 (1943)
68. HAWORTH, W. N., HEATH, R. L., AND PEAT, S., *J. Chem. Soc.,* 55–58 (1942)
69. HASSID, W. Z., AND MCCREADY, R. M., *J. Am. Chem. Soc.,* **63,** 2171 (1941)
70. HASSID, W. Z., CORI, G. T., AND MCCREADY, R. M., *J. Biol. Chem.,* **148,** 89–96 (1943)
71. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.,* **151,** 57–63 (1943)
72. MEYER, K. H., *Advances in Enzymology,* **3,** 109–35 (1943)

73. Mirski, A., and Wertheimer, E., *Biochem. J.*, **36**, 221–31 (1942)
74. Mirski, A., *Biochem. J.*, **36**, 232–41 (1942)
75. Shanks, E., Jr., *J. Med. Assoc. Georgia*, **32**, 80–82 (1943)
76. Wertheimer, E., *Nature*, **152**, 565–66 (1943)
77. Doudoroff, M., Kaplan, N., and Hassid, W. Z., *J. Biol. Chem.*, **148**, 67–75 (1943)
78. Kagan, B. O., Latker, S. N., and Zfasman, E. M., *Biochimia*, **7**, 93–108 (1942)
79. Doudoroff, M., *J. Biol. Chem.*, **151**, 351–61 (1943)
80. Hartt, C. E., *Hawaiian Planters' Record*, **47**, 155–70 (1943)
81. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71–92 (1943)
82. O'Kane, D. J., and Umbreit, W. W., *J. Biol. Chem.*, **142**, 25–30 (1942)
83. Lipmann, F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248–59 (1939)
84. Kalnitzky, G., and Werkman, C. H., *Arch. Biochem.*, **2**, 113–24 (1943)
85. Koepsell, H. J., and Johnson, M. J., *J. Biol. Chem.*, **145**, 379–86 (1942)
86. Doisy, E. A., Jr., and Westerfield, W. W., *J. Biol. Chem.*, **149**, 229–36 (1943)
87. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397–403 (1943)
88. Nachmansohn, D., John, H. M., and Waelsch, H., *J. Biol. Chem.*, **150**, 485–86 (1943)
89. Breusch, F. L., *Enzymologia*, **10**, 165–91 (1942)
*90. Wieland, H., Jennen, R. G., and Schwarze, W., *Ann.*, **548**, 255–70 (1941)
91. Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, **33**, 1980–96 (1939)
92. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149**, 529–41 (1943)
93. Ochoa, S., *J. Biol. Chem.*, **149**, 577–78 (1943)
94. Long, C., *Biochem. J.*, **37**, 215–25 (1943)
95. Ochoa, S., *J. Biol. Chem.*, **151**, 493–505 (1943)
96. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, **137**, 343–56 (1941)
97. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355–62 (1943)
98. Shapiro, B., and Wertheimer, E., *Biochem. J.*, **37**, 102–4 (1943)
99. Breusch, F. L., *Science*, **97**, 490–92 (1943)
100. Wieland, H., and Rosenthal, C., *Ann.*, **554**, 241–60 (1943)
101. Cori, G. T., Ochoa, S., and Cori, C. F. (Unpublished data)
102. Mann, T., *Nature*, **151**, 619–20 (1943)
103. Wendt, G., *Z. physiol. Chem.*, **272**, 152–54 (1942)
104. Kiessling, W., and Meyerhof, O., *Biochem. Z.*, **296**, 410–25 (1938)
105. Ohlmeyer, P., *Z. physiol. Chem.*, **267**, 264–80 (1941)
106. Ohlmeyer, P., and Mehmke, L., *Z. physiol. Chem.*, **272**, 212–16 (1942)
107. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **146**, 673–82 (1942)
108. Utter, M. F., and Werkman, C. H., *J. Biol. Chem.*, **146**, 289–300 (1942)
109. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443–56 (1942)

110. LEHMANN, H., AND POLLAK, L., *Biochem. J.*, **36**, 672–85 (1942)
111. NACHMANSOHN, D., COX, R. T., COATES, C. W., AND MACHADO, A. L., *J. Neurophysiol.*, **6**, 383–95 (1943)
112. FURCHGOTT, R. F., AND SHORR, E., *J. Biol. Chem.*, **151**, 65–86 (1943)
113. SACKS, J., *Am. J. Physiol.*, **129**, 227–33 (1940)
114. BOLLMAN, J. L., AND FLOCK, E. V., *J. Biol. Chem.*, **147**, 155–65 (1943)
115. SACKS, J., *Am. J. Physiol.*, **140**, 316–20 (1943)
116. SACKS, J., AND ALTSHULER, E. H., *Am. J. Physiol.*, **137**, 750–60 (1942)
117. ENGELHARDT, W. A., AND BARCHASH, A. P., *Biochimia*, **3**, 500–21 (1938)
118. BANGA, I., *Z. physiol. Chem.*, **275**, 25–28 (1942)
*119. HUSZÁK, I., *Biochem. Z.*, **312**, 315–29 (1942)
120. COLOWICK, S. P., AND KALCKAR, H. M., *J. Biol. Chem.*, **148**, 117–26 (1943)
121. CORI, C. F., *Biol. Symposia*, **5**, 131–40 (1941)
122. PANY, J., *Z. physiol. Chem.*, **272**, 273–79 (1942)
123. KOSTERLITZ, H. W., *Biochem. J.*, **37**, 318–21 (1943)
124. KOSTERLITZ, H. W., *Biochem. J.*, **37**, 322–26 (1943)
125. KAPLAN, N., AND GREENBERG, D. M., *J. Biol. Chem.*, **150**, 479–80 (1943)
126. NELSON, N., RAPOPORT, S., GUEST, G. M., AND MIRSKY, J. A., *J. Biol. Chem.*, **144**, 291–96 (1942)
127. SACKS, J., *Science*, **98**, 388–89 (1943)
128. NILSSON, R., *Naturwissenschaften*, **31**, 25–35 (1943)
129. ENDERS, C., AND SIGURDSSON, S., *Naturwissenschaften*, **31**, 92–93 (1943)
*130. ENGELHARDT, W. A., AND SAKOV, N. E., *Biochimia*, **8**, 9–36 (1943)
131. TANKÓ, B., *Z. physiol. Chem.*, **276**, 1–16, 17–25 (1942)
132. VANDENDRIESSCHE, L., *Enzymologia*, **10**, 69–78 (1941)
133. KALCKAR, H. M., *J. Biol. Chem.*, **148**, 127–37 (1943)
134. KALCKAR, H. M., *J. Biol. Chem.*, **143**, 299–300 (1942)
135. COLOWICK, S. P., AND SUTHERLAND, E. W., *J. Biol. Chem.*, **144**, 423–37 (1942)
136. MONTIGEL, C., AND VERZÁR, F., *Helv. Physiol. Pharmacol. Acta*, **1**, 115–35 (1943)
137. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **310**, 384–421 (1942)
138. MEYERHOF, O., AND SCHULZ, W., *Biochem. Z.*, **297**, 60–65 (1938)
139. HOUCHIN, O. B., *J. Biol. Chem.*, **146**, 313–21 (1942)
140. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **314**, 149–76 (1943)
141. HERBERT, D., GORDON, H., SUBRAHAMANYAN, V., AND GREEN, D. E., *Biochem. J.*, **34**, 1108–23 (1940)
142. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **314**, 399–408 (1943)
143. UTTER, M. F., AND WERKMAN, C. H., *J. Bact.*, **42**, 665–76 (1941)
144. BINKLEY, F., *J. Biol. Chem.*, **150**, 261–62 (1943)

145. NORBERG, B., *Acta Physiol. Scand.*, **5**, Suppl. XIV (1942)
*146. JANDA, K., AND GÖBELL, O., *Z. Kinderheilk.*, **63**, 524–31 (1942)
*147. GÖBELL, O., AND JANDA, K., *Z. Kinderheilk.*, **63**, 532–37 (1942)
148. COHN, G., AND KOLTHOFF, I. M., *Ind. Eng. Chem., Anal. Ed.*, **14**, 886–90 (1942)
149. JONES, L. T., *Ind. Eng. Chem., Anal. Ed.*, **14**, 536–42 (1942)
150. LEVA, E., AND RAPOPORT, S., *J. Biol. Chem.*, **149**, 47–55 (1943)
151. COURTOIS, J., AND BIGET, P., *Enzymologia*, **10**, 234–38 (1942)
*152. BAILLY, M. C., *Bull. Soc. chim.*, **9**, 314–40, 340–50 (1942)
153. NEUBERG, C., AND LUSTIG, H., *Exptl. Med. Surg.*, **1**, 14–21 (1943)
154. KOSTERLITZ, H. W., AND RITCHIE, C. M., *Biochem. J.*, **37**, 181–86 (1943)
155. HASSID, W. Z., AND MCCREADY, R. M., *Ind. Eng. Chem., Anal. Ed.*, **14**, 683–86 (1942)
156. BARRENSCHEEN, H. K., AND PEHAM, A., *Z. physiol. Chem.*, **272**, 81–86 (1942)
157. BRACHET, J., *Enzymologia*, **10**, 87–96 (1942)
158. HAAS, E., HARRER, C. J., AND HOGNESS, T. R., *J. Biol. Chem.*, **142**, 835–37 (1942)
159. MARENZI, A. D., AND CARDINI, C. E., *J. Biol. Chem.*, **147**, 371–78 (1943)
160. EPSTEIN, J. A., *Biochimia*, **7**, 69–78 (1942)
161. LEPAGE, G. A., AND UMBREIT, W. W., *J. Biol. Chem.*, **147**, 263–71 (1943)
162. LEPAGE, G. A., AND UMBREIT, W. W., *J. Biol. Chem.*, **148**, 255–60 (1943)
163. STONE, W. E., *J. Biol. Chem.*, **149**, 29–41 (1943)
164. KERR, S. E., *J. Biol. Chem.*, **145**, 647–56 (1942)
165. RAPOPORT, S., LEVA, E., AND GUEST, G. M., *J. Biol. Chem.*, **149**, 57–63, 65–69 (1943)
166. BARRENSCHEEN, H. K., AND PEHAM, A., *Z. physiol. Chem.*, **272**, 87–110 (1942)
167. TOROPOVA, G. P., *Biochimia*, **7**, 32–42 (1942)
168. BIELSCHOWSKY, M., AND GREEN, H. N., *Lancet*, **2**, 153–55 (1943)
169. LOOFBOUROW, J. R., *Biochem. J.*, **36**, 737–45 (1942)
170. COOK, E. S., CRONIN, A. G., KREKE, C. W., AND WALSH, T. M., *Nature*, **152**, 474–75 (1943)
171. DAVIES, R., *Biochem. J.*, **37**, 230–38 (1943)
172. GALE, E., AND EPPS, H. M. R., *Nature*, **152**, 327–28 (1943)
173. ERDOS, J., *Science*, **96**, 141–42 (1942)
174. BAER, E., AND FISCHER, H. O. L., *J. Biol. Chem.*, **150**, 213–21 (1943)
175. BAER, E., AND FISCHER, H. O. L., *J. Biol. Chem.*, **150**, 223–29 (1943)
176. NEUBERG, C., *Arch. Biochem.*, **3**, 105–11 (1943)
177. NEUBERG, C., LUSTIG, H., AND ROTHENBERG, M. A., *Arch. Biochem.*, **3**, 33–44 (1943)

*178. Wagner-Jauregg, T., Lennartz, T., and Kuthny, H., *Ber. deut. chem. Ges.*, **74B**, 1513–21 (1941)
*179. Arnold, H., *Ber. deut. chem. Ges.*, **74B**, 1736–40 (1941)
180. Weijlard, J., *J. Am. Chem. Soc.*, **64**, 2279–82 (1942)
181. Barker, G. R., and Gulland, J. M., *J. Chem. Soc.*, 231–32 (1942)
182. Buell, M. V., *J. Biol. Chem.*, **150**, 389–94 (1943)
183. Neuberg, C., and Lustig, H., *J. Am. Chem. Soc.*, **64**, 2722–23 (1942)
184. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1**, 311–18 (1942)
185. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **147**, 41–46 (1943)
186. Tabor, H., and Hastings, A. B., *J. Biol. Chem.*, **148**, 627–32 (1943)
187. Kosterlitz, H. W., *Biochem. J.*, **37**, 321–22 (1943)
188. Kumler, W. D., and Eiler, J. J., *J. Am. Chem. Soc.*, **65**, 2355–61 (1943)

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CARBOHYDRATE METABOLISM

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The principal advances of the past year in carbohydrate metabolism have been in the direction of increased detailed information concerning the mechanisms of carbohydrate utilization and precise chemical data for many of the enzymes and other substances involved.

Glycolysis and Phosphorylations

Knowledge of the first step of the glycolytic mechanism has been greatly increased by a series of fundamental papers from Cori's laboratory (21, 22, 23, 54). Green & Cori (54) have isolated muscle phosphorylase in crystalline form from rabbit muscle. The enzyme constitutes about 2 per cent of the extractable protein—rabbit muscle containing 40 to 80 mg. per 100 gm. The enzyme can be obtained in two forms: (*a*) a crystalline euglobulin, phosphorylase-*a*, which shows 60 to 70 per cent of its maximal enzymic activity without the addition of adenylic acid, and (*b*) a more soluble form, phosphorylase-*b*, which has not been crystallized and which is inactive without the addition of adenylic acid. Phosphorylase-*a* can be transformed to phosphorylase-*b* by an enzyme present in muscle and spleen or by trypsin at pH 6.

Phosphorylase-*a* is a protein with a molecular weight of from 340,000 to 400,000. The dissociation constant with adenylic acid is $1.5 \times 10^{-6}M$ at pH 6.7 and 25°. The enzyme is inhibited by ammonium sulfate, sodium β-glycerolphosphate, and phlorhizin. Phlorhizin inhibition apparently involves a combination of two molecules of the glucoside with one of the enzyme. A competitive inhibition is shown by glucose when the enzyme acts on glucose-1-phosphate as a substrate.

Phosphorylase-*a* apparently contains adenylic acid as a prosthetic group. While the enzymic conversion of phosphorylase-*a* to *b* involves the fission of adenylic acid, free adenylic acid, however, cannot be detected as a product of the conversion. Stimulated muscle contains principally phosphorylase-*b* and it is suspected that the enzyme responsible for the removal of the prosthetic group of phosphorylase-*a* is physiologically important.

Phosphorylase catalyzes the reaction:

glucose-1-phosphate + terminal glucose units ⇆ maltosidic chain units + inorganic phosphate.

The "terminal glucose units" are the end groups of the highly branched glycogen molecule. Polysaccharide synthesis by phosphorylase consists, therefore, in a lengthening of the side chains of glycogen (necessary in small amounts for the *in vitro* reaction to proceed) by addition of glucose units in a 1:4-glucosidic linkage.

The polysaccharide formed by the *in vitro* action of muscle phosphorylase on glucose-1-phosphate [similar in properties to the polysaccharide synthesized by potato phosphorylase and to the amylose fraction from potato starch (60)] is apparently composed of long unbranched chains approximately 200 units in length in which the glucopyranose units are joined in 1:4-α-glucosidic linkages. Such a structure contrasts with the highly branched nature of naturally occurring glycogen. However, it is possible to obtain *in vitro* a polysaccharide resembling glycogen by the simultaneous action of muscle phosphorylase and an enzyme present in heart and in liver (23). It seems, therefore, that the *in vivo* synthesis of glycogen involves the participation of another enzyme in addition to phosphorylase.

Montigel has concluded from a study of the kinetics of glycogen phosphorylation by muscle brei from normal and adrenalectomized animals that desoxycorticosterone is an essential component of the enzyme system involved (103).

There have been several observations pertinent to the effect of potassium ions in phosphorylation reactions. Boyer, Lardy & Phillips (7) have found that potassium ions (in addition to either manganese or magnesium ions) increased the transfer of phosphate from 3-phosphoglycerate to creatine by accelerating the phosphate transfer from 2-phosphopyruvate to the adenylic acid system. These investigators also found potassium ions are necessary for the phosphorylation of creatine during pyruvate oxidation by minced tissues. Kutscher & Krabbenhöft (78) have observed that the four carbon dicarboxylic acids when fed as potassium salts gave rise to as much glycogen deposition as with glucose. While a similar effect is observed with the ammonium salts, the sodium salts are without effect. These results are regarded as indicating an inhibition by sodium ions rather than a stimulation by potassium ions. On the other hand, Sayres, Sayres & Orton (128) found that the administration of sodium chloride with glucose

favored the deposition of glycogen in both the liver and the rest of the carcass. Kossel (69) concludes that the action of manganese and magnesium ions on carboxylase is to prevent the action of inhibitors. The distribution of phosphorylase in various tissues and at various stages of the development of the rat has been studied by Shapiro & Wertheimer (129).

Colowick & Kalckar (20) have shown that hexokinase from yeast catalyzes the reaction:

(*a*). adenosinetriphosphate + hexose → adenosinediphosphate + hexosemonophosphate.

When myokinase from rabbit or frog muscle is also present, Kalckar (66) has shown that the following reaction then occurs:

(*b*). adenosinediphosphate → adenosinetriphosphate + adenylic acid.

The over-all reaction, (*a*) plus (*b*), is represented by equation (*c*):

(*c*). adenosinetriphosphate + 2 hexose → adenylic acid + 2 hexosemonophosphate.

Myokinase is a protein, highly stable to dilute boiling mineral acids and precipitable by trichloracetic acid in the cold without inactivation.

Radioactive phosphorus has again been used to study carbohydrate metabolism in various tissues. Bollman & Flock (6), who studied the penetration and incorporation of isotopic inorganic phosphate into rat muscle, found that the phosphate penetration and uptake into organic combination is the same in resting and exercising muscle. The isotope content of phosphocreatine in the exercising muscle is apparently lower than would be expected if it were derived from inorganic phosphate. Furchgott & Shorr (49) in an admirable study of the same problem in cardiac muscle have distinguished between a readily diffusible, extracellular portion and a poorly diffusible, "bound," intracellular fraction of the total inorganic phosphate. The specific activity of the intracellular fraction determines the specific activities of the organic phosphates. Thus, the isotopic phosphorus content in phosphocreatine in exercising muscle would be expected to be the same as in the resting tissue, since the rate of inorganic phosphate penetration of the tissue is the same in both cases.

Furchgott & Shorr have concluded that their data, as well as those of Bollman & Flock, are entirely in accord with modern concepts of a coupling between phosphorylation and oxidation and the currently

accepted scheme for glycolysis. They ascribe the opposing conclusion reached by Sacks & Altshuler (126) in earlier work, to difficulties involved in the calculation (as contrasted to the measurement) of intracellular inorganic phosphate concentrations.

The demonstration by DuBois & Potter (35) of the presence of a calcium-activated adenosinetriphosphatase in various tissues raises the question of whether the well-known inhibitory effects of calcium on respiration may be interpreted in terms of activation of this enzyme. Breusch (8) holds, however, that the effect of calcium on oxygen consumption is due to the inhibition of the reduction of oxaloacetate, which he regards as a step in hydrogen transport of some tissues.

Meyerhoff & Junowicz-Kocholaty (102) have redetermined equilibrium constants for the reactions catalyzed by the enzymes aldolase, isomerase, and zymohexase (aldolase + isomerase). None of these equilibria is influenced by the presence of inorganic phosphate or by inorganic phosphate plus the oxidizing enzyme of Warburg (1,3-diphosphoglyceraldehyde dehydrogenase) with or without cozymase. There is no evidence, therefore, for the formation of the glyceraldehyde diphosphate postulated by Negelein & Brömel (109) as the precursor of diphosphoglyceric acid. The addition of inorganic phosphate to phosphoglyceraldehyde is believed, then, to involve a loose physical addition product of phosphoglyceraldehyde and inorganic phosphate. The possible formation of a dimeric form of glyceraldehyde-1,3-diphosphate can be discounted inasmuch as a substance of this composition has been synthesized by Baer & Fischer (1) and is not capable of enzymic conversion to 1,3-diphosphoglyceric acid.

Seven of the eleven enzymes involved in the glycolytic system have now been isolated in crystalline form. Warburg & Christian (145) have isolated "zymohexase"[1] from rat muscle as a crystalline protein by fractional precipitation from ammoniacal ammonium sulfate solution. While the crystalline muscle zymohexase does not contain heavy metals and is not sensitive to poisoning by pyrophosphate or cysteine, yeast zymohexase[1] (146), which has not been crystallized, apparently contains a dissociable heavy metal (manganese, iron, cobalt, copper) as a prosthetic group.

Bücher has reported, in a preliminary note, the isolation from yeast of a crystalline protein which catalyzes the interaction of 1,3-diphos-

[1] Warburg's zymohexase is the aldolase of Meyerhoff: the latter uses the term zymohexase for the system aldolase plus isomerase.

phoglyceric acid and adenosinephosphate to yield 3-phosphoglyceric acid and adenosinetriphosphate (14).

Kubowitz & Ott (77) have crystallized the lactic dehydrogenase from rat Jensen sarcoma tissue and compared it with the crystalline dehydrogenase from normal rat muscle. By all criteria used (catalytic activity, dissociation constant, crystal form, rate of destruction by proteolytic enzymes, and ultraviolet absorption spectrum) both proteins were found to be identical or very similar. Both enzymes give rise to similar antienzymes when injected into the rabbit. It is interesting to note that the inhibition of lactic dehydrogenase by its antienzyme was relieved by pyruvic acid, this fact suggesting that the antienzyme and substrate combine with the same group in the enzyme.

Analysis of the serum from normal rats by the optical methods developed by Warburg and his associates shows the presence of zymohexase and isomerase as well as other enzymes of the glycolytic system. In animals having a Jensen sarcoma the quantities of zymohexase and isomerase present in the serum are greatly increased (147). Since aerobic glycolysis is the outstanding metabolic characteristic of tumor tissue, this observation seems of the greatest importance in the metabolic study of malignant tissues.

Relation between Glycolysis and Respiration

Numerous explanations for the effect of oxygen in decreasing the quantity of lactic acid or alcohol formed by many types of living cells have been proposed. Among the causes to which the effect has been ascribed are the following: (*a*) oxidation of intermediary products in the fermentation (anerobic) process; (*b*) resynthesis of carbohydrate from fermentation products; (*c*) decrease in the formation of fermentation intermediates due to the effect of oxygen in maintaining a component of the fermentation enzyme system in an oxidized inactive form. The third hypothesis was supported by the experiments of Laser (79) on rat retina in bicarbonate medium in which it was found that the rate of glycolysis depended upon the oxygen tension and not on the rate of respiration. Craig & Beecher have confirmed these results (25). However, in phosphate buffer, they find that respiration and glycolysis of retina vary inversely with changes in oxygen tension. The extent to which this effect can be ascribed to the specific effect of the buffer is unknown. Laser has shown previously that the absence of carbon dioxide from the medium causes qualitative changes in the respiration of many tissues (80).

Craig & Beecher (26) also found that respiration and glycolysis of rat retina were increased when the carbon dioxide content of the bicarbonate medium was increased from 1 to 5 per cent. Succinate, when added with glucose in a bicarbonate medium, caused increased respiration without affecting glycolysis: in phosphate glucose medium, the addition of succinate is without effect. These facts, as well as earlier observations on tumor glycolysis (148), suggest the advisability of examining these tissues for a carbon dioxide fixing reaction.

A similar reciprocal relationship between glycolysis and respiration obtains in brain cortex (27) and in bone-marrow cells both in the presence and absence of bicarbonate (149). The respiration and glycolysis of the latter tissue has also been studied by Warren & Carter (150) under varying mixtures of oxygen and carbon monoxide to obviate difficulties caused by diffusion and solubility factors at low oxygen tensions. With oxygen–carbon monoxide atmospheres a direct reciprocal relationship also exists between glycolysis and respiration. Since these data would indicate that the Pasteur enzyme described by Stern & Melnick (99, 100, 133) in retina, yeast, and heart muscle has the same affinity for oxygen and carbon monoxide as does the respiratory enzyme, Warren & Carter question the existence of an independent Pasteur enzyme in bone marrow and concur in the opinion of Craig & Beecher that oxygen tension may act on respiration and glycolysis through the mediation of a common agent.

In opposition to earlier reports by various workers, Fuhrman & Field (50) describe experiments which show that iodoacetate inhibits both the respiration and anerobic glycolysis of rat cerebral cortex slices, although the latter is affected first. They concluded that there is no concentration of the drug which will inhibit glycolysis while leaving respiration unaffected, and that experiments with iodoacetate are of questionable value in giving information about the mechanism of the Pasteur effect.

Carbohydrate Oxidation

Tricarboxylic acid cycle.—It becomes increasingly apparent that Krebs' tricarboxylic (citric) acid cycle, or some modification of this scheme, is of basic importance in the mechanism whereby carbohydrate is oxidized in a variety of tissues and species [see Breusch (8), however]. The scheme, as shown in Fig. 1, is in agreement with all the experimental data for pigeon breast muscle, including the data from the isotope experiments in liver (43, 158) which precluded the direct

intermediate formation of citric acid. Krebs (73) has measured the rate of interconversion of the various tricarboxylic acids and has concluded that the *cis*-aconitate formed by condensation of pyruvate and oxaloacetate would, under physiological conditions, yield predominantly isocitric acid so that the observed asymmetric distribution of isotopic carbon is compatible with the formulation of Fig. 1. This point can be definitely established by careful measurements of the amount of isotopic carbon in the second carboxyl group of the isolated α-ketoglutarate.

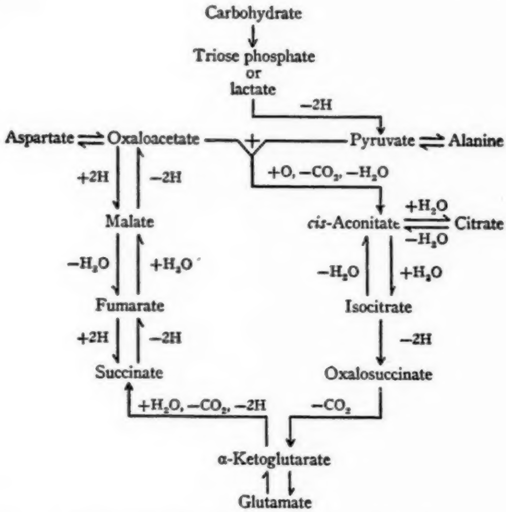

Fig. 1.—The Krebs tricarboxylic acid cycle

Krebs & Eggleston (74) have observed that the polarimetric determination of isocitric, citromalic, and malic acids by the molybdate complex method is invalidated by an effect of citrate itself in increasing the rotation of these complexes. Earlier values for isocitrate must be revised, therefore. Measurements of the equilibrium mixture of the tricarboxylic acids in the presence of liver or muscle aconitase at pH 6.8 (38°, 0.025 *M* phosphate buffer) give 6.2 per cent isocitrate, 4.3 per cent *cis*-aconitate, and 89.5 per cent citrate.

Acetate and acetoacetate.—Evidence has accumulated to indicate that acetic acid and acetoacetic acid are oxidized to carbon dioxide and

water by a series of reactions similar to those postulated for the complete oxidation of pyruvate, i.e., by way of the tricarboxylic acid cycle.

Lynen (93) has presented evidence that the oxidation of acetic acid by yeast occurs by way of the tricarboxylic acid cycle. Acetate condenses with oxaloacetate to form a tricarboxylic acid which is oxidized by way of α-ketoglutarate, succinic semialdehyde, succinate, fumarate, and malate back to oxaloacetate. Virtanen & Sundman (144) have shown the accumulation of citrate when barium acetate is oxidized by yeast—both barium and magnesium ions inhibit the further oxidation of the citrate. Lynen (94a) has also shown that malonate inhibits both the succinic dehydrogenase of yeast and acetate oxidation, and that the inhibition can be removed by the addition of fumarate with the formation of an equivalent quantity of succinate for the fumarate disappearing. These effects could be demonstrated with the sodium salts of the dicarboxylic acids and macerated yeast, as well as with the free acids and suspensions of intact yeast cells. Previous failures to demonstrate malonate inhibition could be ascribed to impermeability of yeast cells to the salts of the various acids involved.

The data with yeast become of more general significance in view of the results of Wieland & Rosenthal (157) on animal tissues. In an earlier paper these workers showed a rapid aerobic utilization of acetate by beef kidney (156). They have concluded now that this utilization probably involves, first, the formation of acetoacetic acid, then the condensation of oxaloacetate and acetoacetate to form a tricarboxylic acid, and the oxidation of this compound by way of the reactions shown in Fig. 1. They have been able to demonstrate an aerobic conversion of added oxaloacetate and acetoacetate to citrate, in which as much as 80 per cent of the added acetoacetate was accounted for by citrate formation when the reaction was carried out in the presence of barium or magnesium ions. The reaction also occurs in beef heart[2] but not in liver. It is of interest to speculate whether such a reaction is concerned in the transient deposition of glycogen observed in adipose tissue (153). Breusch (9) in a preliminary note, without details, has described an enzyme in muscle, kidney, and brain which catalyzes a similar reaction between β-keto acids (including acetoacetic) and oxaloacetate to form citrate.

Ketone bodies.—The conclusion of Krebs & Johnston (75), that acetoacetate is synthesized from acetate and pyruvate by way of aceto-

[2] Barcroft, McNally & Phillipson have noted the utilization of acetate in the isolated perfused heart (5).

pyruvic acid, is supported by the work of Lehninger (81) who has studied the metabolism of this substance. Acetopyruvic acid is ketogenic in the fasting animal and protects against death by insulin hypoglycemia, possibly as the author suggests, by reconversion to pyruvate. The quantitative relationship between acetoacetate and β-hydroxybutyrate in blood and urine of the normal and diabetic human has been studied by Stark & Somogyi (132). Neufeld & Ross (114) report a decrease during exercise in blood ketone bodies of humans on a ketogenic diet, which suggests an utilization of ketone bodies for muscular contraction. An urinary excretion of considerable quantities of choline during ketosis by human diabetics has been noted (86).

Carbon dioxide fixation.—The enzymes involved in the initial reactions of carbon dioxide fixation have been prepared in the form of a cell-free extract of pigeon liver (42). Such extracts contained a heat-sensitive enzyme, activated by manganese ions, which catalyzes the decarboxylation of oxaloacetic acid. This oxaloacetate carboxylase is presumably responsible for the addition of carbon dioxide to pyruvate to form oxaloacetate. It is similar to the enzyme from *Micrococcus lysodeikticus* previously described by Krampitz & Werkman. Krampitz, Wood & Werkman (72) have studied the exchange between $C^{13}O_2$ and the carboxyl group of oxaloacetate during decarboxylation of the latter by their bacterial enzyme preparation. Since oxaloacetate formed from fumarate gave higher values for isotopic exchange of the carboxyl group than synthetic oxaloacetate, they postulated the existence of a "physiological" oxaloacetate, differing in some way (phosphorylation?) from the synthetic form.

The fixation of large quantities of $C^{11}O_2$ in organic form by pigeon liver extracts occurred only in the presence of added pyruvate and fumarate. If pyruvate alone was added, a small quantity of labeled carbon appeared in the pyruvate, however. The detailed mechanism of the fumarate effect, and of the entrance of C^{11} into pyruvate when no fumarate was present, is not entirely certain although several explanations have been suggested (42, 72).

Using acetic, propionic, and butyric acids containing C^{11} in the carboxyl group, Buchanan, Hastings & Nesbett (13) studied liver glycogen when these acids were fed with glucose. The apparent conversion of butyric and propionic acids, but not of acetic acid, to glycogen, was observed.

The recent data of Lorber, Hemingway & Nier (89) showing a fixation of carbon dioxide in the glycogen of the isolated, working,

mammalian heart is of interest since the fixation of appreciable quantities of carbon dioxide has not been shown previously to occur in animal tissues other than liver, although the fixation of relatively small quantities of carbon dioxide in suspensions of pigeon breast muscle has been noted (42).

Hydrogen transport.—Discussion of the mechanism of hydrogen transport from substrate to oxygen during oxidation of carbohydrate has centered about the interpretation of the effects of malonate inhibition.

Fischer, Roedig & Rauch in a paper inaccessible to this reviewer (94) but quoted by Lynen (94a) have apparently suggested that the fumaric hydrogenase discovered by them in yeast is a "degenerated" (*"verdorbene"*) succinic dehydrogenase and that it may participate in yeast metabolism by transporting hydrogen: the hydrogen would presumably go from substrate, triosephosphate or alcohol for example, to cozymase and then to fumarate by way of fumaric hydrogenase and finally to the cytochrome system by an oxidation of succinate to fumarate mediated by succinic dehydrogenase. This is essentially the earlier scheme of Szent-György with fumaric hydrogenase substituted for the "over reduction" of fumarate postulated by him to explain the effect of fumarate in relieving malonate inhibition.

Such a role for fumaric hydrogenase seems unlikely since an examination of the oxidation of ethyl alcohol by yeast (94) discloses that the first phase of this reaction, the aerobic conversion of ethyl alcohol to acetic acid, is not inhibited by malonate although such should be the case if the scheme is correct. On the other hand, the further oxidation of acetic acid to carbon dioxide and water can be almost completely inhibited by malonate as has already been described.

Barron maintains that the assumption of single pathways for carbohydrate oxidation is untenable and postulates a "multiple pathways" scheme of pyruvate oxidation (160). For the direct oxidation of pyruvate he has suggested (160) that the first oxido-reduction in glycolysis (oxidation of phosphoglyceraldehyde by cozymase) is normally followed by a reaction between pyruvate and a flavoprotein to form acetyl phosphate and reduced flavoprotein. The reduced flavoprotein presumably has the properties of Fischer's fumaric hydrogenase, and converts fumarate to succinate, the latter being subsequently oxidized by succinic dehydrogenase. The fumarate arises from malate formed by the reduction of oxaloacetate by cozymase. The oxaloacetate in turn is assumed to be produced by carboxylation of pyruvate.

Under such circumstances, pyruvate would not reduce cozymase but would serve as a source of both oxaloacetate and of acetyl phosphate. This scheme awaits experimental verification. Neither fumaric hydrogenase nor a flavoprotein capable of forming acetyl phosphate from pyruvate has yet been demonstrated in animal tissues. The formation of oxaloacetate from pyruvate and carbon dioxide in skeletal muscle has not yet been demonstrated, although the results of Lorber, Hemingway & Nier (89) suggest the existence of such a reaction in heart muscle. Evidence for the occurrence of a small quantity of oxaloacetate carboxylase activity in skeletal muscle has also been obtained (42).

The paradoxical situation in regard to malonate poisoning of oxaloacetate carboxylase should be pointed out. Oxaloacetate carboxylase (responsible for the carboxylation of pyruvate) is completely inhibited by 0.0025 *M* malonate in cell-free solution (42). On the other hand carboxylation of pyruvate occurs in minced liver in the presence of the same malonate concentration. In heart muscle and pigeon breast muscle the same malonate concentration almost completely inhibits pyruvate utilization in the absence of added dicarboxylic acids so that any appreciable formation of oxaloacetate from pyruvate in those tissues seems excluded. Whether this is a question of differences in the relative concentrations of enzyme and inhibitor in the two tissues or whether there are other factors that modify the effective concentration of the inhibitor, is undecided. A further exploration of these reactions is desirable.

Breusch (8) has made a survey of various tissues in regard to their sensitivity to malonate, their ability to reduce oxaloacetate, their sensitivity to calcium and other factors, and has interpreted his results in terms of an elaborate scheme of hydrogen transport and intermediary carbohydrate metabolism. Since there is as yet little experimental support for many of the details the reader is referred to the original paper.

Question as to the substrate responsible for the oxygen uptake of unsupplemented muscle tissue has been posed by the work of Stadie & Zapp (131). They have studied the respiration of muscle strips, Latapie, blendor, and scissors minces of skeletal muscles of pigeons and cats. In spite of a high oxygen uptake, the decrease in carbohydrate (fermentable reducing substances after acid hydrolysis + lactic acid exclusive of phosphorylated hexoses or hexose derivatives) was insufficient to account for the oxygen uptake. In view of an un-

changed ammonia content, the high respiratory quotient, and absence of ketone body formation, protein and fat seem unlikely as substrates.

Relationship between oxidation and phosphorylation.—The coupling of phosphorylations with the oxidation of pyruvate by heart muscle has been studied by Ochoa (115). He demonstrated that aerobic phosphorylation of phosphate acceptors during pyruvate oxidation is mediated by the adenylic acid system and that three molecules of inorganic phosphate are converted to high-energy-bond phosphate for every primary dehydrogenation (atom of oxygen consumed). The efficiency of conversion of oxidation energy to phosphate bond energy was about 60 per cent. Ochoa has also demonstrated that three moles of high-energy-bond phosphate are apparently formed for each mole of α-ketoglutarate oxidized to succinate and carbon dioxide. An understanding of the mechanisms of these phosphorylations is still lacking, but there is little doubt that the final detailed picture of the tricarboxylic acid cycle must include these reactions. Banga (4) has found that citrate and oxaloacetate are much more effective in affording energy for the phosphorylation of glycogen in extracts of pigeon breast muscle than are pyruvate, fumarate, and aconitate.

Insulin.—When insulin is added to suspensions of pigeon breast muscle, the respiration of the tissue is maintained for a longer period of time than with similar control suspensions. This effect has now been shown (122) to be reflected in the sustained ability of the insulin-supplemented tissue to oxidize pyruvate. When insulin is absent, practically no or very little pyruvate is utilized after 60 to 90 minutes, although the oxygen uptake of the suspension is still of considerable magnitude. It becomes of great interest to ascertain the substrate being used under such circumstances.

The effect of insulin on pyruvate oxidation by muscle suspensions seems to be associated with those steps in the utilization of pyruvate which are catalyzed by either fumarate or oxaloacetate (tricarboxylic acid formation?) since these reactions proceed at a higher rate in insulin-supplemented malonate-poisoned tissue than do the other reactions of the tricarboxylic acid cycle. Wieland & Rosenthal have suggested that insulin may be involved in the condensation reaction between acetoacetate and oxaloacetate (156). If the further suggestion of these authors is confirmed, that pyruvate is metabolized by way of acetate, the results quoted above afford experimental support for such an action of insulin.

However, the *in vitro* relationship between insulin and carbohy-

drate (oxidation) shown by these experiments may be indirect, and there are data from the intact animal that are interpreted to exclude any participation of insulin in pyruvate oxidation. Bueding & Goldfarb (15) have shown that the injection of glucose into normal or psychotic humans gives rise to an increase in blood pyruvate which is not affected by the simultaneous administration of insulin, while the pyruvate-lactate ratio remains constant as has also been found by Stotz & Bessey (136). This is in contrast to observations on depancreatized dogs and diabetic humans (16) in which insulin produces a marked rise in pyruvate after a single glucose administration. However, an increase in normal subjects by insulin administration can be observed if large amounts of glucose are infused constantly (17). Since the rate of pyruvate removal is the same whether insulin is present in normal or excessive amounts or is absent, the increase in blood pyruvate cannot be due to a decreased pyruvate removal. It is therefore concluded that after the administration of glucose to depancreatized dogs, insulin increases the formation of pyruvic acid. This effect is also indicated by the observation that under aerobic conditions insulin increases the phosphorylation of glucose *in vitro* (85), i.e., acts on a stage preliminary to pyruvic acid in carbohydrate metabolism. It would be possible, however, to admit still the possibility of a direct effect of insulin on pyruvate oxidation if it were assumed that the metabolic utilization of pyruvate in the diabetic was qualitatively different from that in the normal subject, the rate of pyruvate removal being the same in both cases.

The effect of insulin on water and electrolyte distribution (137) and on organic phosphate turnover (using isotopic phosphorus as indicator) in muscle (127) and rat liver (67) has been described. The inadvisability of administering glucose to the diabetic is reiterated by Root & Carpenter (123). Mark & Lewis find no evidence of an hepatic inactivation of insulin (95).

Brain and Nervous Tissue

General studies of pyruvate oxidation in brain have been reported by Long (91) and by Simola & Alapenso (130). While a variety of enzymic reactions and effects occur, it is not possible as yet to present any scheme for carbohydrate metabolism in this tissue. Fazekas & Himwich (44) conclude that during partial anerobiosis the brain of the adult cat obtains a part of the energy required for its maintenance

by glycolysis. The glycogen content of various parts of cat and dog brains during the life span has been studied (18).

The electric organ of the electric eel provides a convenient object for studying the changes occurring as a result of the discharge. It now appears that the chain of reactions supplying the energy for the restoration of the resting condition of the organ are identical with or similar to those serving as a source of energy for muscle contraction. The discharge of the organ is associated with the hydrolysis of equivalent amounts of acetylcholine and phosphocreatine (106, 107). An enzyme preparation from the electric organ (and also from brain) synthesizes acetylcholine only in the presence of adenosinetriphosphate (105). Fluoride (which inhibits adenosinetriphosphatase) increases the quantity of acetylcholine formed as do glutamate, succinate, and citrate (108). Cupric ions, iodoacetate, and iodine inhibit acetylcholine formation, while potassium and ammonium ions are without effect. It would appear that the energy for the resynthesis of acetylcholine is derived from phosphocreatine by way of adenosinetriphosphate, creatine phosphate being resynthesized in turn by the energy derived from pyruvate or carbohydrate oxidation.

A relation between glucose content and free acetylcholine of nervous tissue has been noted. Sanz (125) proposes a metabolic scheme linking carbohydrate metabolism with free acetylcholine in rat brain. Welsh (151) has shown that free acetylcholine in cerebral cortex decreases when rats are subjected to low atmospheric pressures and that the decrease is more pronounced during insulin hypoglycemia. He suggests that the decrease in free acetylcholine may account for the decreased excitability of the cortex during conditions of hypoglycemia and anoxia. DuBois & Potter (37) have described an activation of adenosinetriphosphatase by acetylcholine.

Intestinal Absorption

The mechanism of the preferential absorption of glucose from the rat intestine has been studied by Csaky (28) using the 2, 3, 5, and 6 methyl ethers of glucose. The 2, 5, and 6 methyl ethers are absorbed somewhat more slowly while 3-methylglucose (which is not split by intestinal extracts) is absorbed at the same rate as glucose. The data suggest a phosphorylation of glucose during absorption to yield an equilibrium mixture containing principally fructose-1,6-diphosphate. A study of Kjerulf-Jensen (68) of the carbohydrate phosphate esters

of the intestinal mucosa of rats and rabbits when fructose is fed also supports an intermediary phosphorylation of fructose. Kosterlitz has concluded from an analysis of the phosphoric esters present in the liver of rabbits fed galactose that galactose-1-phosphate is present (70). The same investigator suggests that in the fermentation of lactose by yeast galactose-1-phosphate is first formed and converted to the Robison ester by way of glucose-1-phosphate (71).

Since the decrease in serum and urinary phosphorus that is observed when glucose, galactose, and xylose are fed is also observed when the sugars are given intravenously (47), the process of intestinal absorption is apparently not responsible for these changes.

Endocrines and Other Factors Influencing Carbohydrate Metabolism

The ability of the cortin-like material present in urine to cause glycogen deposition in the liver of fasted adrenalectomized rats has been demonstrated (32, 143). While 11-dehydrocorticosterone, corticosterone, and 17-hydroxy-11-dehydrocorticosterone are about equally effective in causing liver glycogen deposition, adrenal steroids without oxygen at C-11 and without the unsaturated ketone group in ring A were inactive (120).

Marx, Anderson, Fong & Evans (97) have shown that preparations of the growth hormone of the anterior pituitary—free of lactogenic, adrenocorticotropic, thyrotropic, and gonadotropic hormones—produce a marked increase in glucose excretion in sucrose-fed, partially depancreatized rats. Russell (124) has studied the apparent increased peripheral utilization of carbohydrate after hypophysectomy in the eviscerated rat in an attempt to decide to what extent adrenal deficiency (mediated through the pituitary) is involved. By comparison of the effects of adrenalectomy, hypophysectomy, and administration of anterior lobe preparations the conclusion was reached that the increased peripheral requirement for carbohydrate by hypophysectomized rats is probably not due to any great extent to diminished adrenal function (gluconeogenesis) and that the pituitary and adrenal hormones can affect carbohydrate metabolism independently.

Hexestrol (4,4′-dihydroxy-α,β-diethyl-dihydrostilbene) causes an atrophy of the testes and a moderate increase in pancreatic insulin while α-methylstilbene causes an increased quantity of pancreatic insulin but is without effect on the testes (58). A decrease in pancreatic insulin during scurvy has been noted (3).

The relation of pantothenic acid to pyruvate metabolism was first indicated in bacteria by Dorfman, Berkman & Koser (32). Later it was shown that diminished pyruvate oxidation occurred in the liver of the pantothenate deficient rat (118) although Teague & Williams (139) concluded that pantothenate was not concerned in glucose, phosphopyruvate, and hexosediphosphate fermentation or pyruvate decarboxylation in yeast extracts and chick brain. Hills (61) now shows that pantothenate increases the aerobic metabolism of pyruvate by washed suspensions of *Proteus morganii* to a greater extent than any of the other substances tested although lactate, succinate, malate, oxaloacetate, α-ketoglutarate, and fumarate had some effect and were all effective in increasing the simultaneous utilization of pyruvate.

Lukens, Dohan & Wolcott (92) have compared the effect of phlorhizin and of insulin in promoting the recovery of cats suffering from experimentally produced pituitary diabetes. The physiological action of the two substances are similar only in their effect on blood sugar, on the hydropic Islands of Langerhans found in diabetic animals and in benefiting early diabetes. These authors suggest that hyperglycemia may play a role in the genesis of diabetes.

General reactions.—Carbohydrate metabolism has been studied during hemorrhagic shock in rats (41). The rise in plasma and whole blood amino nitrogen, in keto acids and lactate, and the hyperglycemia that occurs with a high liver glycogen (but not in starved, suprarenodemedullated, and suprarenalectomized animals) are ascribed to a decrease in hepatic function arising from anoxia and to peripheral effects of anoxia in causing increased protein breakdown and glucose utilization. Anoxia also accounts for the increased blood pyruvate and lactate.

In humans under reduced atmospheric pressures (corresponding to heights of 5,500 to 6,200 meters) the hypoglycemic phase in glucose tolerance tests is shortened or absent (82). There is also an increase in blood pyruvate and lactate and a decreased alkali reserve. Inorganic phosphate in blood and urine decreases with an increase in organic phosphates in the blood. In similar experiments with rats an increase of the hexosemonophosphate content of muscle was found although the glycogen was unchanged. There is evidence for an increased gluconeogenesis from protein in anoxia. The ability to form glycuronates is unimpaired in liver tissue from humans dying from the effects of atmospheric conditions at high altitudes (88). Kruenberg

(76) has also described carbohydrate metabolism during oxygen deficiency.

A local glucogenesis from protein during inflammation has also been postulated (101).

Westerfeld, Stotz & Berg (154) conclude that the increased rate of alcohol metabolism caused by oral pyruvate administration described by them was due to a coupled oxidation-reduction reaction involving the conversion of alcohol to acetaldehyde with the corresponding reduction of pyruvate to lactate, rather than to acetoin formation. Acetoin (155), when fed, is not glycogenic and is excreted in the urine, partly as such, partly as 2,3-butylene glycol. However, Gregory, Ewing & Duff-White (56) find no evidence that pyruvate, insulin, glucose, or insulin plus glucose increases the rate of metabolism of ethyl alcohol. The administration of acetoin and 2,3-butylene glycol accelerates the acetylation of *p*-aminobenzoic acid in rabbits while sodium acetate is without effect (31).

Jacobs (64), in 1937, observed a hyperglycemia in rabbits on the injection of alloxan. Dunn, Sheehan & McLetchie have found (38) that it is possible to cause a typical diabetes in rabbits by alloxan due to a specific degeneration of the islet tissue of the pancreas. A similar effect obtains for rats (40) and dogs (52), but not for humans (11, 12). It is possible to adjust the dose of the substance so that the only pathological lesion of consequence is the effect on the islet tissue. The specificity of action is remarkable.

Gomori reports that liver and kidney contain an alkaline phosphatase with a strict substrate specificity for hexosediphosphate. The enzyme is inactive in absence of magnesium ions and activated by cyanide and inhibited by fluoride (53). Reinecke presents evidence for the formation of a fermentable reducing substance (glucose) by the kidney in eviscerated rats given cortical extracts (120).

Purinton & Schuck (119) have observed an inverse relationship between the tissue concentrations of ascorbic and citric acids and their excretion. The presence of citric acid in bone is paralleled by its occurrence in the eggshells of birds and chickens (140) and also in various tissues in pathological conditions (96). Class & Smith have concluded that the citrate excreted by rats fed sodium bicarbonate is of metabolic rather than of bony origin (19).

It is possible to isolate glycogen from extracts of aphids and of rabbit liver by ultracentrifugation (90).

Greville (57), from a study of intravenous glucose tolerance

curves, has been able to develop an equation expressing the blood sugar concentration as a function of time in the period between five and ninety minutes after intravenous injection of sugar.

Follis & Straight have shown that rats on a completely carbohydrate-free diet grew normally (45).

By comparing lactate formation from glycogen and from fructose-diphosphate by pigeon breast muscle Tanko has concluded that fructose may be metabolized in a qualitatively different fashion from glycogen (138).

The respiratory and carbohydrate metabolism of the malaria parasite (*P. knowlesi*) has been studied by Wendel (152). The presence of various phosphorylated carbohydrate esters in autotrophic bacteria (83) has been described and it has been shown that the adenosinetriphosphate in these organisms is adenosine-3-triphosphate (84).

Studies have been made of corn glycogen (104), the metabolism of the hexitols (65) and lactose (24), the relation between liver glycogenesis and fasting in the rat (98, 141), the distribution of blood lactate between red cells and serum (29), pancreatic amylase (87), and the phosphorolysis of sucrose (33).

Color reactions have been described for aconitic and glutaconic acids (30); methods have been described for the determination of 2,3-diketogulonic acid (117); glucose-1-phosphate and galactose-1-phosphate in liver (70); blood glucose (63); glucose and pyruvate, when together (59); pyruvic acid (48); dihydroxyacetone (142); acetaldehyde (135); diacetyl and acetoin (55, 134); amylase in duodenal contents (46); individual sugars in mixtures of glucose, fructose, mannose, and galactose (10). Methods have been reported for the preparation of the following substances: sodium iodoacetate (51); hexosediphosphate, hexosemonophosphate, and phosphoglyceric acid (34); active zymase extracts and *d*(—)3-phosphoglyceric acid (110, 111); fructose-1,6-diphosphate and fructose-6-monophosphate (112); *d*(—)3-phosphoglyceric acid and *d*(+)2-phosphoglyceric acid (113). The yields of adenosinetriphosphate from muscle can be increased by magnesium anesthetization (36).

Several reviews of the field which have appeared during the year are appended to the bibliography (160–164).

LITERATURE CITED[4]

1. BAER, E., AND FISCHER, H. O. L., *J. Biol. Chem.*, **150**, 213–21 (1943)
2. BAILEY, C. C., AND BAILEY, O. T., *J. Am. Med. Assoc.*, **122**, 1165–66 (1943)
3. BANERJEE, S., *Nature*, **152**, 329 (1943)
*4. BANGA, I., *Z. physiol. Chem.*, **275**, 25–28 (1942); *Chem. Zentr.*, **114**, 417 (1943)
5. BARCROFT, J., MCNALLY, R., AND PHILLIPSON, A., *Nature*, **151**, 304 (1943)
6. BOLLMAN, J. L., AND FLOCK, E. V., *J. Biol. Chem.*, **147**, 155–65 (1943)
7. BOYER, P. D., LARDY, H. A., AND PHILLIPS, P. H., *J. Biol. Chem.*, **149**, 529–41 (1943)
8. BREUSCH, F. L., *Enzymologia*, **10**, 165–91 (1942)
9. BREUSCH, F. L., *Science*, **97**, 490–92 (1943)
10. BRÜCKNER, J., *Z. physiol. Chem.*, **277**, 181–91 (1943)
11. BRUNSCHWIG, A., ALLEN, J. G., GOLDNER, M. G., AND GOMORI, G., *J. Am. Med. Assoc.*, **122**, 966 (1943)
12. BRUNSCHWIG, A., AND ALLEN, J. G., *Cancer Research* (In press)
13. BUCHANAN, J. M., HASTINGS, A. B., AND NESBETT, F. B., *J. Biol. Chem.*, **150**, 413–25 (1943)
*14. BÜCHER, T., *Naturwissenschaften*, **30**, 756–57 (1942); *Chem. Zentr.*, **114**, 2694 (1943)
15. BUEDING, E., AND GOLDFARB, W., *J. Biol. Chem.*, **147**, 33–40 (1943)
16. BUEDING, E., WORTIS, H., AND FEIN, H., *Am. J. Med. Sci.*, **204**, 838–45 (1942)
17. BUEDING, E., FAZEKAS, J. F., HERRLICH, H., AND HIMWICH, H. E., *J. Biol. Chem.*, **148**, 97–104 (1943)
18. CHESTER, A., AND HIMWICH, H. E., *Arch. Biochem.*, **2**, 175–81 (1943)
19. CLASS, R. N., AND SMITH, A. H., *J. Biol. Chem.*, **151**, 363–68 (1943)
20. COLOWICK, S. P., AND KALCKAR, H. M., *J. Biol. Chem.*, **148**, 117–26 (1943)
21. CORI, G. T., AND GREEN, A. A., *J. Biol. Chem.*, **151**, 31–38 (1943)
22. CORI, C. F., CORI, G. T., AND GREEN, A. A., *J. Biol. Chem.*, **151**, 39–55 (1943)
23. CORI, G. T., AND CORI, C. F., *J. Biol. Chem.*, **151**, 57–63 (1943)
24. CORYELL, M. N., AND CHRISTMAN, A. A., *J. Biol. Chem.*, **150**, 143–54 (1943)
25. CRAIG, F. N., AND BEECHER, H. K., *J. Gen. Physiol.*, **26**, 467–72 (1943)
26. CRAIG, F. N., AND BEECHER, H. K., *J. Gen. Physiol.*, **26**, 473–78 (1943)
27. CRAIG, F. N., AND BEECHER, H. K., *J. Neurophysiol.*, **6**, 135–41 (1943)
28. CSAKY, T., *Z. physiol. Chem.*, **277**, 47–57 (1943)
29. DECKER, D. G., AND ROSENBAUM, J. D., *Am. J. Physiol.*, **138**, 7–11 (1942–43)
30. DEFFNER, M., AND ISSIDORIDIS, A., *Biochem. Z.*, **314**, 307–11 (1943)
31. DOISY, E. A., JR., AND WESTERFELD, W. W., *J. Biol. Chem.*, **149**, 229–36 (1943)

[4] References marked with an asterisk have been available in abstract form only and reference to the abstract is given.

32. Dorfman, A., Berkman, S., and Koser, S. A., *J. Bact.*, **43**, 6 (1942)
33. Doudoroff, M., *J. Biol. Chem.*, **151**, 351–61 (1943)
34. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **147**, 41–46 (1943)
35. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **150**, 185–95 (1943)
36. DuBois, K. P., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.*, **147**, 699–704 (1943)
37. DuBois, K. P., and Potter, V. R., *J. Biol. Chem.*, **148**, 451–52 (1943)
38. Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, **1**, 484–87 (1943)
39. Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. Bact.*, **55**, 245 (1943)
40. Dunn, J. S., and McLetchie, N. G. B., *Lancet*, **2**, 384–87 (1943)
41. Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exptl. Med.*, **77**, 397–410 (1943)
42. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771–84 (1943)
43. Evans, E. A., Jr., *Biol. Symposia*, **5**, 157–76 (1941)
44. Fazekas, J. F., and Himwich, H. E., *Am. J. Physiol.*, **139**, 366–70 (1943)
45. Follis, R. H., and Straight, W. M., *Bull. Johns Hopkins Hosp.*, **72**, 39–41 (1943)
46. Free, A. H., and Myers, V. C., *J. Lab. Clin. Med.*, **28**, 1387–98 (1943)
47. Free, A. H., and Leonards, J. R., *J. Biol. Chem.*, **149**, 203–8 (1943)
48. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415–42 (1943)
49. Furchgott, R. F., and Shorr, E., *J. Biol. Chem.*, **151**, 65–86 (1943)
50. Fuhrman, F. A., and Field, J., II, *J. Cellular Comp. Physiol.*, 307–17 (1943)
51. Goldberg, L., *Science*, **98**, 386 (1943)
52. Goldner, M. G., and Gomori, G., *Endocrinology*, **33**, 297–308 (1943)
53. Gomori, G., *J. Biol. Chem.*, **148**, 139–49 (1943)
54. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151**, 21–29 (1943)
55. Greenberg, L. A., *J. Biol. Chem.*, **147**, 11–17 (1943)
56. Gregory, R., Ewing, P. L., and Duff-White, V., *Proc. Soc. Exptl. Biol. Med.*, **54**, 206–8 (1943)
57. Greville, G. D., *Biochem. J.*, **37**, 17–24 (1943)
58. Griffiths, M., *Nature*, **151**, 82 (1943)
59. Haag, E., and Dalphin, C., *Helv. Chim. Acta*, **26**, 246–50 (1943)
60. Hassid, W. Z., Cori, G. T., and McCready, R. M., *J. Biol. Chem.*, **148**, 89–96 (1943)
61. Hills, G. M., *Biochem. J.*, **37**, 418–25 (1943)
62. Horwitt, B. N., and Dorfman, R. I., *Science*, **97**, 337 (1943)
63. Humoller, F. L., *J. Biol. Chem.*, **147**, 281–90 (1943)
64. Jacobs, H. R., *Proc. Soc. Exptl. Biol. Med.*, **37**, 407–12 (1937–38)
65. Johnston, C. H., and Deuel, H. J., Jr., *J. Biol. Chem.*, **149**, 117–24 (1943)
66. Kalckar, H. M., *J. Biol. Chem.*, **148**, 127–37 (1943)
67. Kaplan, N., and Greenberg, D. M., *J. Biol. Chem.*, **150**, 479–80 (1943)
*68. Kjerulf-Jensen, K., *Acta Physiol. Scand.*, **4**, 225–48, 249–58 (1942); *Chem. Zentr.*, **114**, 1903 (1943)

*69. KOSSEL, A. J., *Z. physiol. Chem.*, **276**, 251-67 (1942); *Chem. Zentr.*, **114**, 1785 (1943)

70. KOSTERLITZ, H. W., AND RITCHIE, C. M., *Biochem. J.*, **37**, 181-86 (1943)

71. KOSTERLITZ, H. W., *Biochem. J.*, **37**, 318-20 (1943); **37**, 321-22 (1943)

72. KRAMPITZ, L. O., WOOD, H. G., AND WERKMAN, C. H., *J. Biol. Chem.*, **147**, 243-53 (1943)

73. KREBS, H. A., *Biochem. J.*, **36**, Proc. ix (1942)

74. KREBS, H. A., AND EGGLESTON, L. V., *Biochem. J.*, **37**, 334-38 (1943)

75. KREBS, H. A., AND JOHNSTON, W., *Biochem. J.*, **31**, 772-79 (1937)

76. KRUENBERG, W., *Arch. ges. Physiol. (Pflügers)*, **246**, 171 (1942)

77. KUBOWITZ, F., AND OTT, P., *Biochem. Z.*, **314**, 94-117 (1943)

78. KUTSCHER, W., AND KRABBENHÖFT, F., *Naturwissenschaften*, **31**, 211 (1943)

79. LASER, H., *Biochem. J.*, **31**, 1671-76 (1937)

80. LASER, H., *Biochem. J.*, **36**, 319-35 (1942)

81. LEHNINGER, A. L., *J. Biol. Chem.*, **148**, 393-404 (1943)

*82. LEIPERT, T., AND KELLERSMANN, E., *Z. physiol. Chem.*, **276**, 214-32, 233-50 (1942); *Chem. Zentr.*, **114**, 1290 (1943)

83. LEPAGE, G. A., AND UMBREIT, W. W., *J. Biol. Chem.*, **147**, 263-71 (1943)

84. LEPAGE, G. A., AND UMBREIT, W. W., *J. Biol. Chem.*, **148**, 255-60 (1943)

85. LEVINE, R., FEINSTEIN, R. N., AND SOSKIN, S., *Federation Proc.*, **1**, 50-51 (1942)

86. LINDBERG, O., AND MÖLLERSTROM, J., *Naturwissenschaften*, **31**, 66 (1943)

87. LITTLE, J. E., AND CALDWELL, M. L., *J. Biol. Chem.*, **147**, 229-32 (1943)

88. LODEWIG, P., *Nature*, **151**, 558 (1943)

89. LORBER, V., HEMINGWAY, A., AND NIER, A. O., *J. Biol. Chem.*, **151**, 647-50 (1943)

90. LORING, H. S., AND PIERCE, J. G., *J. Biol. Chem.*, **148**, 35-40 (1943)

91. LONG, C., *Biochem. J.*, **37**, 215-25 (1943)

92. LUKENS, F. D. W., DOHAN, F. C., AND WOLCOTT, M. W., *Endocrinology*, **32**, 475-87 (1943)

*93. LYNEN, F., *Ann.*, **552**, 270 (1942); *Chem. Zentr.*, **114**, 286-88 (1943)

*94. FISCHER, H. O. L., ROEDIG, A., AND RAUCH, K., *Ann.*, **552**, 203 (1942)

94a. LYNEN, F., *Ann.*, **554**, 40-68 (1943)

95. MARK, J., AND LEWIS, R. C., JR., *Bull Johns Hopkins Hosp.*, **72**, 246-48 (1943)

*96. MÅRTENSSON, J., *Kgl. Fijsiograf. Sällskop. hund. Förch*, **11**, 129 (1942); *Chem. Zentr.*, **114**, 170 (1943)

97. MARX, W., ANDERSON, E., FONG, C. T. O., AND EVANS, H. M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 38-39 (1943)

98. MCBRIDE, J. J., *J. Biol. Chem.*, **147**, 333-39 (1943)

99. Melnick, J. L., *J. Biol. Chem.*, **141**, 269–81 (1941)

100. Melnick, J. L., *Science*, **94**, 118–19 (1941)

101. Menken, V., *Am. J. Physiol.*, **138**, 396–407 (1943)

102. Meyerhoff, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71–92 (1943)

103. Montigel, C., *Helv. Chim. Acta*, **26**, 883–905 (1943)

104. Morris, D. L., *J. Biol. Chem.*, **148**, 699–706 (1943)

105. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397–403 (1943)

106. Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, **6**, 383–96 (1943)

107. Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *Proc. Soc. Exptl. Biol. Med.*, **52**, 97–99 (1943)

108. Nachmansohn, D., John, H. M., and Waelsch, H., *J. Biol. Chem.*, **150**, 485–86 (1943)

109. Negelein, E., and Brömel, H., *Biochem. Z.*, **301**, 135–36 (1939); **303**, 132–44 (1939)

110. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1**, 191–96 (1942)

111. Neuberg, C., and Lustig, H., *Arch. Biochem.*, **1**, 311–18 (1942)

112. Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3**, 33–44 (1943)

113. Neuberg, C., *Arch. Biochem.*, **3**, 105–12 (1943)

114. Neufeld, A. H., and Ross, W. D., *Am. J. Physiol.*, **138**, 747–52 (1943)

115. Ochoa, S., *J. Biol. Chem.*, **151**, 493–505 (1943)

*116. Parvé, E. P. S., *Chem. Weekblad*, **40**, 148 (1943); *Chem. Zentr.*, **114**, 2500 (1943)

117. Penney, J. R., and Zilva, S. S., *Biochem. J.*, **37**, 39–44 (1943)

118. Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 237–40 (1942)

119. Purinton, H. J., and Schuck, C., *J. Biol. Chem.*, **148**, 237–43 (1943)

120. Reinecke, R. M., *Am. J. Physiol.*, **140**, 276–85 (1943)

121. Reinecke, R. M., and Kendall, E. C., *Endocrinology*, **32**, 505–8 (1943)

122. Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470–71 (1943)

123. Root, H. F., and Carpenter, T. M., *Am. J. Med. Sci.*, **206**, 234–43 (1943)

124. Russell, J. A., *Am. J. Physiol.*, **140**, 98–105 (1943)

*125. Sanz, M. C., *Arch. ges. Physiol. (Pflügers)*, **246**, 597–607 (1943); *Chem. Zentr.*, **114**, 2699 (1943)

126. Sacks, J., and Altshuler, C. H., *Am. J. Physiol.*, **137**, 750–60 (1942)

127. Sacks, J., *Science*, **98**, 388–89 (1943)

128. Sayers, G., Sayers, M., and Orten, J. M., *J. Nutrition*, **26**, 139–51 (1943)

129. Shapiro, B., and Wertheimer, E., *Biochem. J.*, **37**, 397–402 (1943)

130. SIMOLA, P. E., AND ALAPENSO, H., *Z. physiol. Chem.*, **278**, 57–91 (1943)
131. STADIE, W. C., AND ZAPP, J. A., JR., *J. Biol. Chem.*, **148**, 669–84 (1943)
132. STARK, I. E., AND SOMOGYI, M., *J. Biol. Chem.*, **147**, 731–36 (1943)
133. STERN, K. G., AND MELNICK, J. L., *J. Biol. Chem.*, **139**, 301–23 (1941)
134. STOTZ, E., AND RABORG, J., *J. Biol. Chem.*, **150**, 25–31 (1943)
135. STOTZ, E., *J. Biol. Chem.*, **148**, 585–91 (1943)
136. STOTZ, E., AND BESSEY, O. A., *J. Biol. Chem.*, **143**, 625–31 (1942)
137. SUNDERMAN, F. W., *Am. J. Med. Sci.*, **205**, 102–14 (1943)
*138. TANKO, B., *Z. physiol. Chem.*, **276**, 1–16, 17–25 (1942); *Chem. Zentr.*, **114**, 417 (1943)
139. TEAGUE, P. C., AND WILLIAMS, R. J., *J. Gen. Physiol.*, **25**, 777–83 (1942)
*140. THUNBERG, T., *Kgl. Fijsiograf. Sällskop. hund. Förch.*, **11**, 42, 126 (1942); *Chem. Zentr.*, **114**, 170 (1943)
141. TREADWELL, C. R., TIDWELL, H. C., AND GROFA, B. G., JR., *J. Biol. Chem.*, **149**, 209–15 (1943)
142. TURNER, W. J., KRESS, B. H., AND HARRISON, W. B., *J. Biol. Chem.*, **148**, 581–84 (1943)
143. VENNING, E. H., HOFFMAN, M. M., AND BROWNE, J. S. L., *J. Biol. Chem.*, **148**, 455–56 (1943)
*144. VIRTANEN, A. I., AND SUNDMAN, S., *Biochem. Z.*, **313**, 236 (1942); *Chem. Zentr.*, **114**, 167 (1943)
145. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **314**, 149–76 (1943)
146. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **311**, 209 (1942)
147. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.*, **314**, 399–408 (1943)
148. WARBURG, O., POSENER, K., AND NEGELEIN, E., *Biochem. Z.*, **152**, 309–44 (1924)
149. WARREN, C. O., *J. Cellular Comp. Physiol.*, **19**, 193–209 (1942)
150. WARREN, C. O., AND CARTER, C. E., *J. Biol. Chem.*, **150**, 267–70 (1943)
151. WELSH, J. H., *J. Neurophysiol.*, **6**, 329–36 (1943)
152. WENDEL, W. B., *J. Biol. Chem.*, **148**, 21–34 (1943)
153. WERTHEIMER, E., *Nature*, **152**, 565–66 (1943)
154. WESTERFELD, W. W., STOTZ, E., AND BERG, R. L., *J. Biol. Chem.*, **149**, 237–47 (1943)
155. WESTERFELD, W. W., AND BERG, R. L., *J. Biol. Chem.*, **148**, 523–28 (1943)
156. WIELAND, H., AND ROSENTHAL, C., *Ann.*, **548**, 255 (1941)
157. WIELAND, H., AND ROSENTHAL, C., *Ann.*, **554**, 241–60 (1943)
158. WOOD, H. G., WERKMAN, C. H., HEMINGWAY, A., AND NIER, A. O., *J. Biol. Chem.*, **139**, 483–84 (1941)

REVIEWS

159. BARRON, E. S. G., "Mechanisms of Carbohydrate Metabolism," *Advances in Enzymology*, **3**, 149–90 (1943)

160. Barron, E. S. G., "Some Considerations on the Application of Biological Oxidation-Reduction Reaction Systems to the Study of Cellular Respiration," *Biol. Symposia*, **10**, 27–70 (1943)

161. Dorfman, A., "Pathways of Glycolysis," *Physiol. Revs.*, **23**, 124–38 (1943)

162. Krebs, H. A., "The Intermediary Stages in the Biological Oxidation of Carbohydrate," *Advances in Enzymology*, **3**, 191–252 (1943)

163. Meyer, H. K., "Chemistry of Glycogen," *Advances in Enzymology*, **3**, 109–36 (1943)

164. Soskin, S., "Storage and Significance of Tissue Glycogen in Health and Disease," *Arch. Internal Med.*, **71**, 219–29 (1943)

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FAT METABOLISM

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Digestion and Absorption

Hoagland & Snider (1) have continued their series of excellent studies on the digestibility of different fats. Seven fats having a wide range of digestibility were fed to rats as 5 and 15 per cent of the diet and it was again found that the poorly digested fats had higher digestive coefficients when fed at elevated levels, but no constant relationship existed between melting point and digestibility. A comparison of the absorption of egg oil and cod liver oil when administered by stomach tube to rats has shown that the former is somewhat more readily absorbed (2).

In measurements of fat excretion by normal children, four to twelve years of age, Williams *et al.* (3) have found that with fat intakes of between 62 and 113 gm. per day, utilization varied from 96 to 98 per cent. About 50 per cent of the fat in the feces was present as soaps and 30 per cent as nonsaponifiable matter.

In a study of the utilization of fats by herbivora, Paul & McCay (4) have found that guinea pigs can readily utilize diets containing 30 per cent fat, but elaidic acid is not as efficiently digested as it is in rats. Both rabbits and sheep digest hydrogenated fats (Crisco) as well as liquid fats (cottonseed oil), and rabbits and guinea pigs can utilize castor oil. Whitson *et al.* (5) have developed a technique for measuring fat digestibility in chickens which makes use of barium sulfate as an indicator of the ratio of feed to feces.

The importance of the stearic acid content of fat upon digestibility has been stressed by Hoagland & Snider (6) who fed mixtures of fatty acids and pure triglycerides in olive oil to rats. Stetten (7) has presented evidence that a hydrocarbon, *n*-hexadecane, containing heavy hydrogen is efficiently absorbed by the rat. Some of the compound was deposited as such in the tissue lipids and some was converted to fatty acids.

An interesting study of the adaption of digestive enzymes to the type of food ingested has been made by Grossman, Greenberg & Ivy (8) who maintained rats on constant diets and then studied the pancreatic content of trypsin, amylase, and lipase. When a high carbohydrate diet was fed there was a pronounced increase in pancreatic amylase and a decrease in trypsin. A high protein diet gave an increase in trypsin and some increase in lipase. However, a high fat diet resulted in no alteration of either trypsin or lipase. Schmidt & Thannhauser (9) carefully purified an alkaline phosphatase from the intestinal mucosa and found that while phosphopyruvic, pyrophosphoric adenylpyrophosphoric, and diphenylphosphoric acids were hydrolyzed, the phosphatides were completely resistant.

Pancreatectomy produces an impairment in fat absorption, but Vermeulen, Owens & Dragstedt (10) have found that in some depancreatized dogs as much as 75 per cent of the ingested fat is absorbed. They further report that the temporary lipemia following fat ingestion is abolished following pancreatectomy and is not restored by the administration of active pancreatic juice or raw pancreas. In human subjects deprived of the external pancreatic secretion by operative procedures incident to disease the administration of pancreatic enzymes (11, 12) or an increase in the dietary protein (11) increased the amount of fat absorbed. The decrease in fat absorption by rats following adrenalectomy, reported by Bavetta and his co-workers, appears to be restricted to fatty acids of eight or more carbons (13).

The reliability of the chylomicron count in the determination of blood lipids and in studying rates of fat absorption has been confirmed by Cooper & Lusk (14). The lipemia that follows fat ingestion in dogs has been found by Hahn (15) to be immediately abolished if heparin is given intravenously. When added to lipemic blood *in vitro,* heparin was without effect.

Adlersberg & Sobotka (16) have found that in sprue, the ingestion of fat and vitamin A does not result in an increase in the blood levels of these substances. When lecithin was given at the same time there was a moderate rise in the serum lipids of normal controls and of subjects with sprue (17). The serum lipids were not altered when lecithin alone was fed. In patients with gastric cancer and hepatic cirrhosis, Rekers, Abels & Rhoads (18) found that the ingestion of large amounts of fat did not cause a rise in fecal fat. However, abnormal absorption was observed in a gastrectomized subject and one with atrophic gastritis. A possible explanation for the more complete absorption of

fats that slowly pass from the stomach is suggested by the work of Tidwell (19) who found that the absorption of a small amount of fat is necessary for the initiation of the hormonal control of gastric activity.

In self-selection studies, following ligation of the bile duct, rats showed a tendency to avoid fat foods and to increase their intake of carbohydrate foods (20). This implied importance of bile in fat absorption was not borne out by the findings during a period of obstructive jaundice in a human subject without pancreas secretion but receiving oral pancreatic enzyme therapy (12). The result of this study suggested that pancreatic enzymes are active in the absence of bile and are relatively more important than bile. Selye (21) has reported that bile does not play any important role in the absorption of the steroid hormones (desoxycorticosterone, progesterone, and testosterone) from the intestinal tract.

Synthesis and Deposition

Depot fat.—Fractionations of human depot fat by Cramer & Brown (22) permitted the isolation of pure myristic, palmitic, stearic, and oleic acids as methyl esters. The presence of tetradecenoic and hexadecenoic acids was demonstrated for the first time. Oleic and linoleic (8 to 11 per cent) were the principal unsaturated C_{18} fatty acids present, but they were found along with isomeric octadecenoic and octadecadienoic acids. The presence of arachidonic acid (0.3 to 1.0 per cent) in human fat was confirmed.

Chemical analyses of fats from turkeys and chickens of various breeds show no significant differences (23). Female turkeys always have a higher muscle fat content than males (24) just as in most other species. Loeb (25) has found that under certain conditions the administration of estradiol benzoate to male rats slightly increases the total body fat.

Fatty acids rich in deuterium were found by Stetten & Grail (26) to disappear much more rapidly from liver fat (half-life, 2.6 to 2.8 days) than depot fat (half-life 5 to 6 days). The presence or absence of choline had no effect on the rate of turnover of the deutero fatty acids indicating a synthesis of fat from carbohydrate in both cases.

Odd numbered carbon-chain fatty acids were well utilized by rabbits (27) and goats (28) and in the latter were deposited in large quantity in the depot fat. It is not clear whether the fed acids only

or homologues of these were also deposited. Undecylic acid is deposited in large amounts when fed to rats but there is no evidence of a higher homologue being synthesized (29). The latter type of chain lengthening would be involved in the claimed conversion of palmitic to stearic acid (30). Visscher (31) has found that the fatty acids stored by the rat on a diet very low in fat are the same as on a diet containing 5 per cent palmitic acid with or without the addition of linoleic acid.

Hypophysectomy in the rat usually leads to a reduction in the appetite and hence body fat, but Samuels *et al.* (32) have found that this is not the case if the operated animals are force-fed to equal the food intake of litter mate controls.

Liver fat.—It is still uncertain what agents are responsible for the lipotropic action of protein and pancreas extracts. Channon and his co-workers (33) have investigated fourteen pure amino acids, among which only tryptophane was found to possess lipotropic action on the fat and cholesterol types of fatty livers. Glutamic acid was found to exert a lipotropic action on cholesterol livers.

Gavin *et al.* (34) have compared various lipotropic factors. Choline was found to be effective against thiamin fatty livers, partly effective against cholesterol fatty livers, and without effect on biotin fatty livers. Both inositol and lipocaic were active against biotin fatty livers. Inositol showed no activity against thiamin fatty livers but the addition of other B vitamins permitted inositol to become lipotropic. This finding has been confirmed (35). It is uncertain whether the lipotropic activity of lipocaic is due to its content of inositol and choline or choline precursors. The observation that inositol, but not lipocaic, is effective in preventing cholesterol fatty livers suggests the presence of an unknown lipotropic substance in addition to the two mentioned above. Dam & Kelman (36) have made the very interesting observation that the blood phospholipids of vitamin E deficient chicks are increased when vitamin E or lipocaic is included in the diet. The inference drawn is that the vitamin E deficient chick is lacking in the active principle of lipocaic and that the formation of this substance depends upon the presence of vitamin E in the diet.

As a result of studies of simultaneous mineral and choline deficiencies on the liver fat of the rat (37) and the influence of choline deficiency on the fat content of regenerating liver (38), Handler suggests that the development of fatty livers in this deficiency can proceed only when all other dietary factors will permit the growth of the whole rat

rather than merely growth of the liver. Handler & Bernheim (38) state that

> The effect of deficiencies of members of the vitamin B complex in preventing the appearance of fatty livers due to choline deficiency is the result of an impairment of the over-all metabolism rather than some specific defect in the metabolism of the liver.

Parenteral administration of lipocaic prevents the accumulation of liver fat produced by the ketogenic anterior pituitary hormone in the fasting guinea pig (39). The hypophysectomized and depancreatized dog develops a fatty liver as rapidly as the depancreatized animal, and this is also prevented by lipocaic therapy according to the findings of Dragstedt's group. Lipocaic administration prior to operation resulted in a normal fat content in the livers of patients with cancer of the gastrointestinal tract, and this appeared to be due to the inositol content of the lipocaic (40).

Ralli & Rubin (41) conclude, from studies in depancreatized dogs and dogs deprived of their external pancreatic secretion, that fatty livers which develop under these conditions are due to a state of protein depletion caused by impaired digestion and the presence in meat of some substance capable of producing fatty infiltration. Dried meat powder, which is presumably more easily digested than raw meat, produced no fatty infiltration of the liver unless meat juice or inositol was fed with it. Allen and his co-workers (42) have found that the total loss of pancreatic juice by a pancreatic fistula did not produce fatty infiltration of the liver as in the insulin-treated depancreatized dog. They conclude that lipocaic is not lost in the pancreatic juice. This work is not in harmony with that of Ralli & Rubin (41) or of others (43) who have reported the development of fatty livers when pancreatic juice is excluded from the intestinal tract. It is probable that the action of lipocaic will not become clear until more attention is given to the over-all nutritional status of the experimental animals as has been suggested by Handler.

Synthesis of fat from carbohydrate.—Supporting the theory that the specific dynamic action of carbohydrate is due to the energy wasted in intermediary metabolism, Ring (44) has found that the S.D.A. of thiamin plus glucose is twice as great as that of glucose alone. Thiamin does not influence the S.D.A. of fat nor does it have any effect alone. It is considered by Ring to be necessary for the conversion of carbohydrate to fat and the S.D.A. is assumed to be the energy required for this conversion of carbohydrate into a form suitable for storage.

The relative importance of the liver and of extra-hepatic tissues in the conversion of carbohydrate to fat remains uncertain. Recently there has been a revival of interest in glycogen deposition in adipose tissue (45, 46, 47). Recovery-feeding of fasted rats with carbohydrate leads to marked glycogen storage in fat tissue as measured histologically (46, 47). Brown fat stores more glycogen than white fat and appears to store as much as liver although there is no correlation between the glycogen content of brown fat and that of liver (46). Lactose differs from most carbohydrates in that no glycogen is stored in the adipose tissue when it is fed. These histological studies agree with the chemical study reported earlier by Tuerkischer & Wertheimer (45). More recently Mirski (48) has shown that adipose tissue phosphorylates glycogen, thus giving the Cori ester. Ordinary white adipose tissue of rats lacks phosphoglucomutase while the brown interscapular fatty tissue of rats contains this enzyme. An enzyme solution which synthesizes glycogen from glucose-1-phosphate may be obtained from adipose tissue by Cori's procedure. Tissue slice experiments with adipose tissue show that glycogen-rich fat has a very high respiratory quotient as compared with that of starvation fat. The addition of glucose increases the respiratory quotient of the latter. The conclusion is reached that the glycogen-rich, high respiratory quotient, adipose tissue slices are converting glycogen or added glucose into products of low oxygen content, probably fatty acids.

Utilization

Lipolytic and dehydrogenating enzymes.—Kelly (49, 50), in extensive investigations of the lipolytic activity of bovine mammary gland tissue, has observed that the tissue of undeveloped glands contains only traces of active lipase, but when developed by pregnancy significant amounts of this enzyme are present. The lipolytic activity has been attributed to the secretory tissue itself which normally has an acid reaction with the secretory cell areas of the lactating glands showing the highest hydrogen ion concentration. The butyric esterase content of serum of mice was measured by Khanolkar & Chitre (51) who found that in a cancer-resistant strain the esterase activity was lower than in two cancer-susceptible strains.

Progress in the study of the fatty acid dehydrogenase of cattle liver by Annau, Eperjessy & Felszeghy (52) has shown that this enzyme dehydrogenates lecithin, with methylene blue as the hydrogen acceptor, when xanthine or hypoxanthine is present as an activator.

The enzyme also dehydrogenates stearic and palmitic acids, but does not act on oleic acid. Another interesting observation made by these investigators was that the season of the year influenced the quantity of enzyme so markedly that demonstrable amounts were found only during the winter. Shapiro & Wertheim (53) have studied a fatty acid dehydrogenase in adipose tissue, but in this case it was found that adenylic acid and inorganic phosphate were required for activity. The enzyme was active on the longer chain fatty acids and phospholipids, but was inactive on neutral fat. Liver, muscle, heart, and testes were also reported to contain this enzyme.

In examining the specificity of a β-hydroxybutyric acid dehydrogenase extracted from pig heart, Lang (54) found that it acted readily on higher homologues of β-hydroxybutyric acid.

Ketosis.—The modern view which regards ketosis as a physiological mechanism in which a special type of fat metabolism comes into play was suggested independently by Drury (55, 56) and Blixenkrone-Moeller (57) and is now widely accepted. Briefly outlined (58), the ketone bodies are supposed to be produced by the oxidation of fatty acids by the liver and are carried in the blood stream to the extrahepatic tissues where they are utilized as a source of energy. This transformation of fatty acids is a supplementary mechanism operating when there is a shortage of carbohydrate for fuel.

In experiments on man and guinea pigs, Neufeld & Ross (59) have confirmed the earlier observations of Drury and his co-workers (56, 60) that during ketosis the utilization of ketone bodies by the muscles and their production by the liver are increased. The intact organism or a dependent limb have been shown to utilize ketone bodies at a rate proportional to their concentration until high blood levels are reached. Shaw & Peterson (61) have now demonstrated that as much as 88 per cent of the oxygen consumption of the perfused bovine mammary gland may be due to the burning of β-hydroxybutyric acid when the concentration of the latter is maintained above 28 mg. per cent. Kravel & Gibson (62) have found that liver and muscle do not oxidize acetone appreciably *in vitro* at concentrations as high as 317 mg. per cent.

Roberts & Samuels (63) have reported that the onset of fasting ketosis is more rapid in rats which have been receiving a high fat diet than in those previously fed a high carbohydrate diet. However, the marked lipemia that follows diethylstilbestrol administration to ducks is not accompanied by an increase in blood ketone bodies (64).

Stark & Somogyi (65, 66, 67) have made a series of interesting observations on the ketone bodies. Their concentration was found to be twice as high in human plasma as in the red blood cells. In plasma β-hydroxybutyric acid is the larger in percentage, but in the corpuscles acetoacetic acid constitutes the bulk of the ketone bodies. With their method they found small amounts of ketone bodies in the urine of all normal and diabetic patients. In healthy persons glucose feeding during hunger ketosis causes the distribution ratio [total ketone bodies in cells]/[total ketone bodies in plasma] to become inverted and in the postabsorptive state it is below unity.

The β ratio [100xβ-hydroxybutyric acid]/[total ketone bodies] decreases until several hours after glucose feeding when it becomes zero and the ketone bodies are represented by acetoacetic acid alone. In the urine the β ratio decreases more rapidly and increases sooner than in the blood. Patients with mild diabetes respond after sugar as do normals while severe diabetics show no change in the distribution or β ratios unless they are given effective doses of insulin.

Studies of twenty diabetics in mild and severe states of ketosis by Martin & Wick (68) have shown that there is a low ketonuria, largely due to non-threshold acetone, when the blood acetone bodies are below 20 mg. per cent. A general relationship between blood levels and urinary excretion exists, but the excretion of ketone bodies may be impaired in cases of renal failure. This study also indicates a poor correlation between the blood level of ketone bodies and the carbon dioxide combining power.

In order to produce a fasting ketosis in female goats and dairy cows, Forbes (69) found that it was necessary for the animals to be in good condition and either approaching parturition or in early lactation. It is interesting to note that in spite of such difficulties involved in producing an experimental ketosis the prevalence of an idiopathic ketosis in cows, especially in the farm areas of the eastern states, has become a serious problem to the dairy industry.

Special oxidation mechanisms.—During this year there has been a considerable collection of information regarding possible mechanisms of fatty acid oxidation *in vivo*. Improved methods for the determination of such substances as the volatile fatty acids (70), oxalic acid (71), acetaldehyde (72), acetic acid (73), and acetopyruvic acid (74) will greatly aid in the elucidation of these oxidation processes. The metabolism of possible two carbon intermediates, especially acetic acid, has received wide attention. Lehninger (75) has confirmed the isola-

tion of acetic acid as the 2,4-dinitrophenylhydrazide from liver. Further evidence that triacetin is readily metabolized by rats, when as much as 20 per cent of the diet is fed as such, has been presented by McManus, Bender & Garrett (76). Dye & Marsters (77) found that intravenously administered acetic, butyric, caproic, caprylic, and capric acids were as well utilized in eviscerated dogs as in normal dogs; the respective utilizations were: 60, 43, 39, 71, and 20 per cent. Since a marked rise in metabolism occurred in both groups, it was assumed that the utilization was due, in part at least, to oxidation. There appears to be ample evidence that most of the tissues of the body can utilize the short chain acids, particularly acetic acid.

Several significant contributions indicate a close relationship between the metabolism of acetic acid and carbohydrate. Virtanen & Sundman (78) have studied the influence of various cations on the formation of citric acid from acetic acid by yeast and have noted an accelerating influence of both barium and magnesium. Slade & Werkman (79) have observed that cell suspensions of *Aerobacter indologenes* in the presence of glucose will condense acetic acid to succinic acid. The carbon to carbon linkage which takes place involves the methyl carbons of the two acetic molecules. Lynen (80, 81) has studied the metabolism of acetic acid added to an aqueous suspension of impoverished brewers' yeast and has presented evidence that this compound enters the citric acid cycle. The mechanism postulated is that carbon dioxide together with acetic acid forms oxalacetic acid. The oxalacetic acid in turn is converted to citric acid, then to ketoglutaric acid, and finally passes through the usual four-carbon dicarboxylic acid stages. Malonic acid inhibits this system and it has been assumed that the inhibition is at the point where succinic acid is converted to fumaric acid because fumarate additions will restore the cycle. Breusch (82) has postulated an enzyme system in animal tissues that is very similar to the one described by Lynen for yeast. In this case an active enzyme preparation (citrogenase) that has been isolated from muscle, kidney, and brain tissue will catalyze the formation of citric acid from β-keto acids. The postulated cycle involves the condensation of oxalacetic acid with a two carbon fragment which is split from a β-keto acid. Citric acid is formed and the conventional conversions of this cycle follow. Muñoz & Leloir (83) have isolated an enzyme preparation from liver that catalyzes the oxidation of the lower fatty acids (especially butyrate, crotonate, valerate, hexanoate, heptanoate, and octanoate). Approximately one molecule of oxygen is

taken up for every molecule of butyric acid that disappears and it is believed that β-hydroxybutyric acid is the major product formed. Inorganic phosphate, fumarate, cytochrome-*c*, adenylic acid, and magnesium were found to be necessary components of this system. There appears to be a striking similarity between the components of this fatty acid oxidation system and systems concerned with oxidation of carbohydrate.

A tagging experiment by Buchanan, Hastings & Nesbett (84), in which acetic, propionic, and butyric acids containing radioactive carbon in the carboxyl groups were fed to rats, is in some respects inconsistent with the above postulations of fatty acid oxidation. The acids were given together with glucose, and liver glycogen was isolated and examined for the presence of radioactive carbon. When corrected for the carbon that could come from carbon dioxide, the liver glycogen contained no excess radio-carbon following acetic acid feeding. About 3 per cent and 1 per cent of the fed radio-carbon was found when propionic and butyric acids respectively were fed. If acetic and pyruvic acids follow the same general pathway of oxidation, as would be inferred from the studies of Lynen and of Breusch, an excess deposition of radio-carbon in the liver glycogen following acetic acid feeding would be expected. The small butyric acid conversion to glycogen may be explained on the basis of a partial ω-oxidation to succinic acid.

The metabolism of several other possible fatty acid intermediates has been studied. Oxalic acid has been found to be utilized to some extent by rats (71). Acetoin is utilized by rats, but is not converted into glycogen (85). Acetopyruvic acid is reported to be ketogenic in fasting rats, but an adequate control with alkali was not carried out, so the results should not be considered conclusive (74). Glycolic acid forms neither glycogen nor ketone bodies in the fasted rat (86). Glyoxylic acid is ketogenic in the fed rat as indicated by a rise in blood ketone bodies following its administration. As a result of the latter observation it has been suggested that glyoxylic acid may be the two carbon fragment formed by the β-oxidation of fatty acids (86).

Keil (87) has found that the feeding of triglycerides of synthetic branched-chain fatty acids gives an increase in the ether-soluble acids in the urine. This is mainly true of the ethyl branched-chain fatty acids which are inefficiently attacked by the body in comparison with the methyl branched acids. The triglyceride of γ-ethylcaprylic acid gave rise to the production of β-ethylenanthic acid, presumably through β-oxidation (88).

Artom & Swanson (89) fed bromine-substituted fatty acids and found that they caused fatty infiltration of the liver in an inverse relation to the rate of bromine excretion, which excretion rate was probably a measure of the liberation of bromine by oxidation of the acid; the liver fatty acids were largely free of bromine. It is suggested that the removal of the halogen is preliminary to further oxidation and if this is by β-oxidation the products would be physiological and easily disposed of. If, on the other hand, initial removal of the halogen occurs at positions not normally attacked by β-oxidation, the products may be such as are used with difficulty and would accumulate in the liver. The acids which caused the greatest increases in liver fat contained bromine in positions which would not be physiologically attacked by β-oxidation and those causing the smallest increases had bromine where it would be susceptible to β-oxidation.

Non-Caloric Functions

Essential fatty acids.—Symptoms of essential fatty acid deficiency in dogs have been reported by Hansen & Wiese (90) who fed puppies a low fat diet and observed a generalized flaky desquamation with coarse dry hair at about three months of age. These skin changes were prevented by the inclusion of lard in the diet. The iodine values of the blood lipids, particularly the acetone-soluble fraction, were markedly lowered in the deficient animals. Bailey (91) measured the curative properties of a large number of fish oils and found them to be decidedly inferior to methyl linolate in alleviating the symptoms of essential fatty acid deficiency in rats. Twenty-four seed oils of known linoleic and linolenic acid content were assayed for their relative potency in curing rat acrodynia by Anthony *et al.* (92). Curative powers were in direct proportion to their linoleic acid content and it was observed that frequently linolenic acid reduced this effect.

Nutritive value of different fats.—There are still conflicting reports concerning the nutritive value of different fats. It is possible that many of these apparent discrepancies can be given a rational explanation if such variables as the composition of the basal diet, and the relative food intakes of the experimental animals (appetite effects) are given careful consideration.

In an investigation by Nitschke (93), rats which had been fed a fat-free diet were subjected to a four-day starvation period and then re-fed a diet containing 30 per cent of one of four fats that were being studied. Cooking fat was found to promote the greatest gain, followed

by olive oil and two synthetic fats, called artificial fats A and B. Boer & Jansen (94) again report that for growth butter fat is superior to other fats (margarine, peanut oil, olive oil, and others) even though these latter fats were fortified with vitamins A and D. The importance of the type of carbohydrate included in the basal ration on the elicitation of a superior growth-promoting effect by butter has been stressed by Boutwell and his co-workers (95, 96). Slight differences in growth rates of rats receiving butter fat or corn oil were found only when lactose was in the diet. If the lactose, either in the form of milk or the pure compound, was diluted with dextrose the differences were decreased. When dextrose, sucrose, dextrin, or corn starch supplied all of the carbohydrate the differences were abolished. Not all investigations have shown a superior growth-promoting effect of butter fat. Deuel, Movitt & Hallman (97) measured the growth rates of rats receiving olive oil, peanut oil, soybean oil, or butter fat incorporated in mineralized skim milk and found no significant differences, although there was a slight tendency for the rats receiving butter fat to grow the most rapidly. If diacetyl (an artificial butter flavor) was added to the vegetable fats a slight increase in growth rate was observed. These authors conclude that with an adequate vitamin intake, all fats studied were of equal nutritional value and that differences, when observed, were merely reflections of appetite effects. Euler, Euler & Säberg (98) have also concluded that when adequately fortified with vitamins, margarine is not inferior in growth-promoting qualities to butter. An excellent summary of the relative nutritive value of different fats has been prepared by the Committee on Fats of the National Research Council (99). In conclusion they state:

> It seems clear therefore, that experiments may be designed in which growth and reproductive performance can be materially altered by changing the type of fat in the diet. However, with not more than 20 per cent of fat in the usual mixed diet the difference between fats sold under the same trade names may often be greater than the difference between unlike fats of diverse origins. Furthermore, upon repetition, factors, of which the experimenter is unaware, may bring about a reversal in results.

In recent years there has been an accumulation of evidence tending to show that the general physiological responses of several body processes, particularly growth and lactation, are improved by the ingestion of fat in excess of the essential fatty acid requirements. However, Heywang (100) has found the hatchability of white leghorn

eggs was not statistically influenced by diets containing from 0.8 to 8.8 per cent fat.

Miscellaneous relationships.—In a past study, French (101) found that among the particular diets studied, the most efficient utilization of calcium by rats was obtained when the diet contained approximately 5 per cent fat. This has been erroneously quoted by reviewers (102, 103) as evidence that some fat in the diet enhances calcium absorption, although the experiments actually showed that with diets containing about 5, 14, 28, and 45 per cent fat (oleo oil), respectively, there was a progressive decline in calcium absorption. French & Elliott (104) have extended this study to completely adequate diets containing from 0.28 to 39.29 per cent fat. When fed in equicaloric amounts which provided approximately equal intakes of nitrogen, calcium, and phosphorus, it was again found that increases in the fat content of the diet slightly decreased the acidity of the intestinal contents and the retention (absorption) of calcium. The total absence of pancreatic enzymes from the gastrointestinal tract of a human subject, with the resulting marked loss of fat in the feces, was found to be accompanied by an abnormally high fecal excretion of calcium (12). Bunkfeldt & Steenbock (105) studied the effect of dietary fat (cottonseed oil) on bone calcification in rats receiving diets completely deficient in vitamin D. They observed that when the diet was very low in phosphorus the calcification of the femur was lowered by fat. When phosphorus was present at optimal or above optimal levels, dietary fat increased calcification. However, if the Ca: P ratio was above 4 the fat effect was not found.

Although dietary fat has been found to be necessary for the normal absorption of vitamin A, Reifman, Hallman & Deuel (106) have not observed any relationship between the rates of absorption of neutral fat and vitamin A. Adlersberg & Sobotka (17) and Slanetz & Scharf (107) have observed a pronounced increase in vitamin A absorption when phosphatides were simultaneously administered. This effect may be due, in part at least, to an antioxidant action similar to the recently demonstrated effect of tocopherols on vitamin A utilization. An adequate explanation for the very rapid gastrointestinal destruction of vitamin A has been proposed by Hove (108) who found an active lipoxidase in the stomach but not in the intestines of rats. This enzyme, which catalyzes the destruction of carotene in the presence of methyl linolate, was inhibited by α-tocopherol.

The effects of fat rancidity and of antioxidants on the availability

of dietary essentials have been stressed by several groups of investigators. Pavcek & Shull (109) have found a marked lability of biotin to the action of rancid fat, and Strohecker & Buchholz (110) have pointed out that fats exert a destructive influence on ascorbic acid when water is present, while in the dry condition the vitamin is not affected. Biological evidence that hydrogenation of natural fats containing tocopherols offers protection of vitamin E activity has been obtained by Miller (111), who found that nutritive value, as measured by the number of rats born and weaned, was higher for hydrogenated than for unhydrogenated vegetable oils.

Clausen, Barnes & Burr (112) have found that in purified diets not containing yeast or some active antioxidant, the fat may develop high peroxide values in as short a time as two weeks. The pure B vitamins which are known to be essential in the diet of rats did not exert any protective influence, but yeast was significantly antioxidative. György & Tomarelli (113) have observed an antioxidant effect of yeast and also of oat, wheat, corn, rice bran, liver extract, molasses, and milk sugar residue. A number of the pure B vitamins were studied, but *p*-aminobenzoic acid was the only active substance found in this group of compounds. Unpublished results from one of our laboratories (114) show that neither *p*-aminobenzoic acid nor sulfanilamide exerts any antioxidative action upon the fat (lard) of experimental diets. It should be mentioned that György's group utilize the change in iodine number of the fatty acids as an index of the progress of oxidation. Significant oxidative destruction may take place before measurable changes in iodine number are noticed, so that the comparison of rates of fat oxidation by this method does not reliably indicate the termination of the induction period which is generally considered the most significant chemical index of the keeping quality of a fat. The inadequacy of iodine number changes as a measurement of fat oxidation rates has been discussed by Lea (114a). György, Stiller & Williamson (115) have found that the antioxidative activity of sulfhydryl compounds requires the presence of water and postulate that these compounds may exert a protective action *in vivo*. Barnes *et al.* (116) have concluded that the antioxidants which naturally occur in the fat tissues cannot be synthesized by the rat but must be supplied in the diet. Furthermore, it would appear that the tocopherols are the only substances that are effective in this respect. Houchin (117) has conducted further studies on the recently established phenomenon of increased oxygen consumption by muscle tissue

from vitamin E deficient animals. An attempt to rule out a non-biological antioxidant action as a factor in this increased oxygen consumption was made, but to the reviewers it seems that an enzymatic oxidation of fatty acids has not been completely excluded. Oxidation of fatty acids at double bonds such as takes place in the rancidification process has never been observed in living tissues. The observations of Hove (108), who found an enzyme similar to the lipoxidase of soybeans in extracts of rat stomach and liver, provide some indirect evidence that this type of oxidation may take place. Further studies on the kinetics, activation, and inhibition of the lipoxidase of plant tissues, particularly from soybeans, have been made by several investigators (118 to 121). Balls, Axelrod & Kies (121) have noted that the enzyme acts only on the "essential fatty acids" and have made the interesting postulate that the role of the essential acids in animal metabolism may be one of intermediate catalysis in some form of tissue oxidation.

A number of other non-caloric activities of fats have been observed. Dam (122) has found that the exudative diathesis which is characteristic of vitamin E deficiency in chicks occurs when diets contain 5 per cent cod liver oil, slightly rancid cod liver oil, or a mixture of fresh and completely rancid cod liver oil. It is concluded that rancidity does not contribute to these symptoms, but highly unsaturated acids seem to be necessary. Fish body and liver oils contain a substance which is effective in reducing the blood pressure of experimentally produced hypertension in rats (123). The effectiveness of these oils is increased by oxidative procedures which destroy vitamin A. Kamen (124) administered acrolein to dogs, cats, and rabbits and produced symptoms of burn shock. It is postulated that acrolein produced by the breakdown of fats may be a contributing factor in burn shock. However, as shock can be produced at temperatures much lower than are necessary for the formation of acrolein, this substance cannot be generally involved. Roffo & Roffo (125) have reported that oxidized fats fed to rats over a period of two years cause the development of gastric cancer. The diverse effects of solvents upon tumor production by carcinogenic agents is, for the most part, still unexplained. Dickens & Weil-Malherbe (126) and Merelli (127) have both confirmed the work of others that benzopyrene becomes less carcinogenic when dissolved in solvent-extracted fat from rat carcasses. In these studies the method of fat extraction removed the phospholipids and when phospholipids alone were used as the solvent for benzopyrene, normal carcinogenic activity was observed (127). It is possible that oxidation

of the fat produced pro-oxidants which in turn destroyed the carcinogen and that phospholipids prevented this effect through an antioxidant action. This type of carcinogen destruction by rancidifying fat has been given recent confirmation (111). In addition it has been shown that many carcinogenic hydrocarbons are pro-oxidants themselves and the postulate has been advanced that their carcinogenic action is effected through a destruction of phospholipids (128). Another interesting relationship between fat and experimental cancer has been shown by Wicks & Suntzeff (129) who found that two to ten days after a single local application of methylcholanthrene the ratio of total lipid to protein nitrogen of the epidermis of mice was reduced. In normal rats Wynn, Giddings & Haldi (130) have found that the ingestion of a high fat diet raised the fat content and lowered the protein content of the skin within one week.

New investigations (131, 132) have confirmed the observations of Johnson, Freeman, and their co-workers, that the ingestion of a high fat diet increases red blood cell destruction, presumably by the fatty acids and soaps present in duct lymph. They have also demonstrated the presence in lipemic blood of an agent which increases the fragility of red blood cells (133). When an adult drinks one pint of 32 per cent cream the blood serum becomes injurious to the erythrocytes (132). Loewy *et al.* (131) in dogs and Josephs *et al.* (134) in normal infants have noted an increased output of urobilin in the feces when fats were ingested in increased amounts. This is indicative of extra blood destruction and it is interesting to speculate as to the part which excessive fat ingestion or abnormal fat absorption may play in certain human anemias. It has also been suggested that this fat effect in its milder form may be a normal mechanism for disposing of an excess of red blood cells.

Other Lipids

Phospholipids.—Perhaps the most serious handicap in the study of the metabolic functions of lipids is the inadequacy of available analytical methods. This is particularly true in the case of the phospholipids. Marenzi & Cardini (135) have made a careful re-examination of lipid extraction, lipid phosphorus, and phospholipid choline methods and have introduced certain modifications of their own. In addition, they have presented values for total phospholipid, choline phospholipid, lecithin, cephalin, sphingomyelin, and choline in normal human plasma. Artom & Fishman (136) have also given considerable

attention to phospholipid methods and have developed new modifications for the determination of several fractions. The validity of measurements of α and β isomeric forms of phospholipids has been contested in the past on the basis that acid or alkali treatment causes a rearrangement of glycerophosphoric acid. Contrary to this, Burmaster (137) has found that alkali treatment of α and β glycerophosphate alone or in mixtures with choline, serine, ethanolamine, and inositol did not cause any shift of the isomers. Woolley (138) has continued his investigations on the structure of phospholipids and has reported the isolation of a new inositol-containing phospholipid, lipositol, from soybeans. Analysis of this compound shows it to contain 16 per cent inositol, 15.5 per cent carbohydrate (galactose), 8.3 per cent *d*-tartaric acid, and 23.6 per cent oleic acid.

An interesting influence of the diet upon the composition of tissue phospholipids has been shown by Artom & Fishman (139, 140, 141) who found that rats receiving a purified laboratory diet had less choline-containing phospholipids in their livers than rats that had been fed a mixed stock diet. Although the composition of the two diets was completely different, these authors have related this effect to the casein of the experimental ration. Supplementing of the casein with choline, ethanolamine, *dl*-serine, glycine, *l*-cystine, or *dl*-methionine did not raise the choline-containing phospholipids to the level observed on the stock diet. However, there was a slight indication that choline supplements caused an elevation in this phospholipid fraction in weanling rats. The distribution of phospholipids in cancer tissue has been investigated by two groups of workers (142, 143). In one of these studies (143) nuclei separations were made and the conclusion was drawn that sphingomyelin was not present in the nuclei, but the proportion of lecithin at this site was higher than in the whole cells.

Fishler *et al.* (144) injected inorganic P^{32} into hepatectomized dogs and then measured the recovery of radio-phosphorus in the phospholipids of several tissues. From these data it is apparent that the plasma phospholipids synthesized in the kidney and intestine are not available to the plasma. Five hours after the injection of plasma containing radioactive phospholipids, Zilversmit *et al.* (145) found that 51 to 58 per cent of the phospholipid P^{32} was still in the plasma. The liver contained the next largest amount of this labeled lipid. The rate of turnover of different phospholipid fractions was measured by Hunter & Levy (146), and it was found that radio-phosphorus was slower to enter the sphingomyelins than the other phospholipids of the

liver. In the kidney the rates of P^{32} turnover were approximately the same for all the phospholipids. Continuing their studies on the lipid metabolism of the fasting mouse, Hodge *et al.* (147) have noted that the P^{32} turnover of blood phospholipids passes through a maximum on the second and third day of starvation. The liver phospholipids were separated into so-called α- and β-fractions (not necessarily representing isomeric moieties) and with the exception of α-cephalin which remained constant, these fractions all showed a sharp increase in P^{32} turnover, and reached a maximum on the second fasting day.

Special tissue lipids.—In a series of three papers, Dimter (148, 149, 150) has reported the isolation of a hydrocarbon resembling squalene from mammalian dermoid cysts and fetal and mature livers, but not from blood serum or depot fat. It is postulated that this hydrocarbon is a precursor of cholesterol. It was mentioned above that another hydrocarbon, *n*-hexadecane, may be absorbed from the intestinal tract and deposited in adipose tissue (7). Schuwirth (151) has made a very complete analysis of the lipid constituents of the human spinal cord; included are values for fat and cholesterol, ether-soluble glycerophosphatides, sphingomyelins, cerebrosides, and gangliosides. Klenk and his co-workers have continued their studies on the structure of the sugar-containing lipids and have isolated hexacosanoic acid from the fatty acids of brain cerebrosides (152). In another publication Klenk & Rennkamp (153) have reported the composition of three fractions of cerebrosides and gangliosides from cattle spleens. The cephalin fraction of human brain has been found to increase with age at the expense of other lipid fractions (154). Kaucher *et al.* (155) have determined the "essential" lipids in a wide variety of tissues and species. Confirming a similar relationship previously demonstrated for the phospholipids they found the essential lipid concentration to be related to the extent and variety of the physiological activities of the various tissues studied.

Cholesterol.—There have been few significant contributions in the field of cholesterol metabolism. Sperry & Brand (156) have introduced certain modifications and have described conditions for accuracy in the determination of blood cholesterol by the acetic anhydride method. Bloch & Rittenberg (157) have described a method whereby deuterium may be introduced into the side chain and ring of cholesterol. This deuterium-containing cholesterol was utilized by Bloch, Berg & Rittenberg (158) in studying the biological conversion of cholesterol to cholic acid. An anastomosis was made between the

gall bladder and the kidney pelvis in a dog. The labeled cholesterol was given intravenously and deuterium was found in the excreted cholic acid. Three days after its administration, the highest concentration of labeled cholesterol was found in the lungs followed by the liver. None was deposited in the brain or spinal cord. Rosenheim & Webster (159) have found cholestenone in dog feces after feeding brain; they take the view that it may be an intermediate in the conversion of cholesterol to coprosterol. Coprosterol formation in the intestinal tract is completely inhibited by succinyl sulfathiazole, but this interference appears not to be due to the action of the drug on either bacteria or protozoa (160). Heinrich & Mattill (161) have reported that vitamin E deficiency in rats results in a marked increase in brain cholesterol and a lesser increase in muscle cholesterol. Total muscle lipids were unaffected. Foldes & Beecher (162) have made the interesting observation that the depth and duration of ether and barbiturate anesthesia are greatly increased by a previous injection of cholesterol.

Pathological Conditions

Blood lipids.—Glomset & Bollman (163) have found that the plasma lipids in dogs are depressed by a high lard diet and are temporarily elevated by a high egg yolk diet. They concluded that the total phospholipids and cholesterol are the most reliable indices of the variability of the plasma lipids. Peters & Man (164) studied the serum lipids in the postabsorptive state in normal persons and found that the ratios of cholesterol to lipid phosphorus and of free to total cholesterol were constant. Confirming others they found (165) that the serum cholesterol rises when the thyroid is removed and falls with the administration of active thyroid substance. However, they note that normal concentrations of serum cholesterol may be found both in patients with hyperthyroidism and thyroid deficiency. They report (166) that serum cholesterol is frequently elevated in patients with renal disease when there is edema associated with hypoproteinemia but the hypercholesterolemia cannot be correlated with any single feature of the disease. Thomas (167) reports a case of severe nephrotic hyperlipemia (edema and hypoproteinemia) in a child in which the concentration of total plasma lipids reached a maximum of 6.6 gm. per 100 cc., and that of total cholesterol 2.0 gm. per 100 cc. Periods of elevation of the plasma lipid concentrations paralleled periods of clinical exacerbation of the disease. In connection with work we have discussed earlier (132), it is interesting that the blood of this

patient had a distinct tendency to hemolyze spontaneously *in vitro*. In the dog and monkey bilateral nephrectomy or ureteral ligation causes a marked and progressive increase of total lipid and cholesterol of the serum (168) which is apparently not related to the lack of food, water, or other factors incident to the uremia. The injection of massive doses of insulin into normal human subjects does not significantly alter the level of blood lipids (169).

Josephs (170) has found a reduction of the vitamin A, carotene, and total lipid contents of the serum of children with pneumonia. During convalescence the serum lipids of older children rose to a concentration above normal and remained high for some time. Carotene but not vitamin A serum levels could be correlated with the lipid level. LeWinn & Zugerman (171) gave 100 gm. of fat to twenty patients with acne vulgaris and observed that the subsequent changes in blood cholesterol were not different from normal controls.

Hepatic cirrhosis.—The old idea that a reciprocal relation always exists between the glycogen and fat content of the liver is known to be false. In addition there has been an incorrect interpretation of the protection afforded by a high carbohydrate diet against liver damage by chloroform. As a result of these misconceptions attempts to improve the resistance of the liver to injury from hepatotoxic agents have been restricted to the use of glucose administration. By biopsy studies Ravdin's group (172) has shown that glycogen is not accumulated in the liver with glucose administration alone unless the total caloric intake is in excess of basal requirements. Even though such conditions are attained there is no guarantee that the glycogen deposition will reduce the liver fat content which is now recognized to be important in protecting against liver damage. The well-known lipotropic agents are either protein components or associated with protein foods and Ravdin *et al.* (172) find that the same type of diet (20 per cent protein, 74 per cent carbohydrate, and 6 per cent fat) which protects the liver from damage in the rat is effective in restoring to normal the fat and glycogen contents of the human liver. Treadwell, Tidwell & Grafa (173) have found that there is no correlation between the glycogen and fat of dietary fatty livers. In the rats having fatty livers there appeared to be an increased rate of glycogenolysis during fasting and a decreased glycogenesis following a standard dose of glucose. Deuel & Davis (174) have also observed a decreased glucose tolerance in rats with dietary fatty livers; the effect is more marked in females than males. Apparently not all functions are impaired in

fatty livers, for Szego & Barnes (175) report that the liver mechanism for the detoxification of estrone is not inhibited by dietary fat accumulating in the liver.

There is considerable evidence that in fatty livers, of some types at least, damage occurs more readily than in non-fatty livers. Chaikoff *et al.* (176) find that the deposition of excess fat in the liver may alone so damage this organ in the dog that a cirrhosis is produced. The same appears to be true in rats (177). But does not the very presence of an excessive amount of liver fat indicate that another factor is operative, namely, the dietary deficiency which permitted this deposition of fat?

Obesity.—In an excellent review containing many new observations, Newburgh (178) finds that obese subjects expend more energy to perform a measured amount of work than do normal persons. Evidence is presented that although heredity influences body build, the latter does not determine the presence of obesity. According to Newburgh, when obesity is a hereditary disease in man, it is the excessive appetite and not the fat deposits which is inherited. He believes that in no case is it justifiable to attribute obesity to hypopituitarism. Although hypophysectomized rats may retain normal amounts of depot fat if force-fed (32), they generally have a decreased appetite and they lose weight. Hetherington (179) finds no obesity after hypophysectomy, but an excess of fat is quickly deposited in the depots as a result of hypothalamic lesions even in the absence of the hypophysis.

There is a strain of so-called "congenitally obese" yellow mice in which Newburgh (178) believes one is dealing with a hereditary bulimia. His contention that obesity is always due to taking in more food than is used up cannot be denied so that the immediate cause is an appetite which is not coordinated with the energy requirement. However, in some cases an attractive possibility is that the ultimate cause of obesity rests in abnormal fat depots which accept fat as readily as in the normal but release it less easily, with the result that the fat depots slowly increase. Salcedo & Stetten (180) present evidence which favors such a view. They fed deutero fatty esters to adult "congenitally obese" mice and compared the results with similar data on normal mice. They conclude that the proportion of the dietary fatty acids directly stored in the depots of the obese mice is normal. The turnover of depot fatty acids was much slower than in the normal mice, so they attribute the obesity to a retarded catabolism of the depot fatty acids. Another theoretical possibility is that in some cases

the obese organism may convert carbohydrate, which is the source of most depot fat, to fatty acids at an abnormally high rate. Along this line liver biopsies show a striking relation between the obesity of the patient and the lipid content of the liver (172). There is a reduced glucose tolerance in many obese individuals, but Newburgh (181) has shown that in 77 per cent of those adult obese hyperglycemic patients who were willing to undergo weight reduction the glucose tolerance became normal.

Lipidosis.—By repeated injections of lecithin (182) and ether-insoluble lipids of beef brain (183) Tompkins has reproduced in part some of the pathology of the lipid storage diseases. Bossa (184) has offered a classification of the lipidoses based on their chemical characteristics. One type is the Niemann-Pick or Tay-Sach's variety. It is characterized by the accumulation of phospholipids in the internal organs, particularly in the spleen. A second type is found in Gaucher's disease where there are deposits of the cerebroside, cerasin, in the internal organs, especially in the spleen. In the third type where the disease of Hand-Christian-Schueller is the most prominent representative, the lipid accumulation consists of cholesterol and its esters. A common characteristic of the lipidoses is the general disturbance of the lipid metabolism and the decreased utilization of some fraction. Garb (185) points out that xanthoma diabeticorum is not a disease entity like some xanthomas but the symptom and the result of a lipemia secondary to diabetes.

Krause (186) has made an extensive examination of the changes in the bone marrow lipids of cats which were made anemic by frequent bleeding. In anemia the bone marrow lost total lipid due to a reduction in the neutral fat fraction. The concentrations of phospholipid, free fatty acid, cholesterol, and cholesterol-free non-saponifiable substances increased. The ratios of free to bound cholesterol, and of choline to phosphorus, showed no significant changes, and there were no changes in the iodine number and mean molecular weights of the fatty acids. The proposal was made that the lipemia which follows acute hemorrhage may be due to the transport of lipids to and from the marrow.

A number of interesting reviews that appeared during the year are cited at the end of the bibliography (187 to 195).

LITERATURE CITED

1. Hoagland, R., and Snider, G. G., *J. Nutrition*, **25**, 295–302 (1943)
2. Reder, R., *Poultry Sci.*, **21**, 528–31 (1942)
3. Williams, H. H., Endicott, E. N., Shepherd, M. L., Galbraith, H., and Macy, I. G., *J. Nutrition*, **25**, 379–87 (1943)
4. Paul, H., and McCay, C. M., *Arch. Biochem.*, **1**, 247–53 (1943)
5. Whitson, D., Carrick, C. W., Roberts, R. E., and Hague, S. M., *Poultry Sci.*, **22**, 137–41 (1943)
6. Hoagland, R., and Snider, G. G., *J. Nutrition*, **26**, 219–25 (1943)
7. Stetten, D., Jr., *J. Biol. Chem.*, **147**, 327–32 (1943)
8. Grossman, M. I., Greenberg, H., and Ivy, A. C., *Am. J. Physiol.*, **138**, 676–82 (1943)
9. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369–85 (1943)
10. Vermeulen, C., Owens, F. M., Jr., and Dragstedt, L. R., *Am. J. Physiol.*, **138**, 792–96 (1943)
11. Rekers, P. E., Pack, G. T., and Rhoads, C. P., *J. Am. Med. Assoc.*, **122**, 1243–45 (1943)
12. Lake, M., Cornell, N. W., and Harrison, H. E., *Am. J. Med. Sci.*, **205**, 118–22 (1943)
13. Bavetta, L. A., *Am. J. Physiol.*, **140**, 44–46 (1943)
14. Cooper, R. R., and Lusk, H., *Am. J. Digestive Diseases*, **9**, 395–96 (1942)
15. Hahn, P. F., *Science*, **98**, 19–20 (1943)
16. Adlersberg, D., and Sobotka, H., *Gastroenterology*, **1**, 357–65 (1943)
17. Adlersberg, D., and Sobotka, H., *J. Nutrition*, **25**, 255–63 (1943)
18. Rekers, P. E., Abels, J. C., and Rhoads, C. P., *J. Clin. Investigation*, **22**, 243–48 (1943)
19. Tidwell, H. C., *Proc. Trans. Texas Acad. Sci.*, **25**, 33–38 (1941)
20. Richter, C. P., and Birmingham, J. R., *Am. J. Physiol.*, **138**, 71–77 (1942–43)
21. Selye, H., *Endocrinology*, **32**, 279–81 (1943)
22. Cramer, D. L., and Brown, J. B., *J. Biol. Chem.*, **151**, 427–38 (1943)
23. Nutter, M. K., Lockhart, E. E., and Harris, R. S., *Oil & Soap*, **20**, 231–34 (1943)
24. Harshaw, H. M., Kellogg, W. L., Rector, R. R., and Marsden, S. J., *Poultry Sci.*, **22**, 126–36 (1943)
25. Loeb, H. G., *Proc. Soc. Exptl. Biol. Med.*, **51**, 330–32 (1942)
26. Stetten, D., Jr., and Grail, G. F., *J. Biol. Chem.*, **148**, 509–15 (1943)
27. Hock, A., *Ernährung*, **6**, 278–81 (1941); *Chem. Abstracts*, **37**, 6014 (1943)
28. Appel, H., Böhm, H., Keil, W., and Schiller, G., *Z. Physiol. Chem.*, **274**, 186–205 (1942); *Chem. Abstracts*, **37**, 6713 (1943)
29. Visscher, F. E., *J. Biol. Chem.* (In press)
30. Stetten, D., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 329–45 (1940)
31. Visscher, F. E., and Corley, R. C., *J. Biol. Chem.*, **147**, 291–95 (1943)
32. Samuels, L. T., Reinecke, R. M., and Bauman, K. L., *Endocrinology*, **33**, 87–95 (1943)
33. Channon, H. J., Mills, G. T., and Platt, A. P., *Biochem. J.*, **37**, 483–92 (1943)

34. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275–79 (1943)
35. Forbes, J. C., *Proc. Soc. Exptl. Biol. Med.*, **54**, 89–90 (1943)
36. Dam, H., and Kelman, E. M., *Science*, **96**, 430 (1942)
37. Handler, P., *J. Biol. Chem.*, **149**, 291–93 (1943)
38. Handler, P., and Bernheim, F., *J. Biol. Chem.*, **148**, 649–54 (1943)
39. Julian, O. C., Clark, D. E., Van Prohaska, J., Vermeulen, C., and Dragstedt, L. R., *Am. J. Physiol.*, **138**, 264–68 (1943)
40. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Proc. Soc. Exptl. Biol. Med.*, **54**, 157–58 (1943)
41. Ralli, E. P., and Rubin, S. H., *Am. J. Physiol.*, **138**, 42–49 (1942–43)
42. Allen, J. G., Vermeulen, C., Owens, F. M., Jr., and Dragstedt, L. R., *Am. J. Physiol.*, **138**, 352–56 (1943)
43. Popper, H. L., and Necheles, H., *Proc. Soc. Exptl. Biol. Med.*, **51**, 63–65 (1942)
44. Ring, G. C., *Am. J. Physiol*, **138**, 487–90 (1943)
45. Tuerkischer, E., and Wertheimer, E., *J. Physiol.*, **100**, 385–409 (1941)
46. Eger, W., *Arch. path. Anat.* (Virchow's), **309**, 607–24 (1942); *Chem. Abstracts*, **37**, 5114 (1943)
47. Shanks, E., Jr., *J. Med. Assoc. Georgia*, **32**, 80–82 (1943)
48. Mirski, A., *Biochem. J.*, **36**, 232–41 (1942)
49. Kelly, P. L., *J. Dairy Sci.*, **26**, 385–99 (1943)
50. Kelly, P. L., *Proc. Assoc. Southern Agr. Workers, Ann. Convention*, **43**, 108–9 (1942); *Chem. Abstracts*, **37**, 1751 (1943)
51. Khanolkar, V. R., and Chitre, R. G., *Cancer Research*, **2**, 567–70 (1942)
52. Annau, E., Eperjessy, A., and Felszeghy, O., *Z. Physiol. Chem.*, **277**, 58–65 (1942); *Chem. Abstracts*, **37**, 5426 (1943)
53. Shapiro, B., and Wertheim, E., *Biochem. J.*, **37**, 102–4 (1943)
54. Lang, K., *Z. Physiol. Chem.*, **277**, 114–16 (1942); *Chem. Abstracts*, **37**, 5427 (1943)
55. Barnes, R. H., and Drury, D. R., *Proc. Soc. Exptl. Biol. Med.*, **36**, 350–52 (1937)
56. Drury, D. R., *Calif. Western Med.*, **45**, 45–48 (1936)
57. Blixenkrone-Moeller, N., Cited by Lundsgaard, E., *Harvey Lect.*, **33**, 65–87 (1938)
58. MacKay, E. M., *J. Clin. Endocrinology*, **3**, 101–10 (1943)
59. Neufeld, A. H., and Ross, W. D., *Am. J. Physiol.*, **138**, 747–52 (1943)
60. Drury, D. R., Wick, A. N., and MacKay, E. M., *Am. J. Physiol.*, **134**, 761–68 (1941)
61. Shaw, J. E., and Peterson, W. E., *J. Biol. Chem.*, **147**, 639–43 (1943)
62. Kravel, K. K., and Gibson, R. B., *J. Iowa State Med. Soc.*, **33**, 183–84 (1943)
63. Roberts, S., and Samuels, L. T., *J. Biol. Chem.*, **151**, 267–71 (1943)
64. Mirsky, I. A., Grayman, I., and Nelson, N., *Proc. Soc. Exptl. Biol. Med.*, **51**, 363–64 (1942)
65. Stark, I. E., and Somogyi, M., *J. Biol. Chem.*, **147**, 319–25 (1943)
66. Stark, I. E., and Somogyi, M., *J. Biol. Chem.*, **147**, 721–30 (1943)
67. Stark, I. E., and Somogyi, M., *J. Biol. Chem.*, **147**, 731–36 (1943)

68. Martin, H. E., and Wick, A. N., *J. Clin. Investigation*, **22**, 235–41 (1943)
69. Forbes, R. M., *Cornell Vet.*, 33, 27–47 (1943)
70. McClendon, J. F., *Federation Proc.*, **2**, 66 (1943)
71. Adolph, W. H., and Liang, C.-C., *J. Biol. Chem.*, **146**, 497–502 (1942)
72. Stotz, E., *J. Biol. Chem.*, **148**, 585–91 (1943)
73. Caselli, P., and Ciaranfi, E., *Biochem. Z.*, **313**, 11–30 (1942); *Chem. Abstracts*, **37**, 5096 (1943)
74. Lehninger, A. L., *J. Biol. Chem.*, **148**, 393–404 (1943)
75. Lehninger, A. L., *J. Biol. Chem.*, **149**, 43–45 (1943)
76. McManus, T. B., Bender, C. B., and Garrett, O. F., *J. Dairy Sci.*, **26**, 13–23 (1943)
77. Dye, J. A., and Marsters, R. W., *Federation Proc.*, **2**, 11 (1943)
78. Virtanen, A. I., and Sundman, J., *Biochem. Z.*, **313**, 236–46 (1942); *Chem. Abstracts*, **37**, 5102 (1943)
79. Slade, H. D., and Werkman, C. H., *Arch. Biochem.*, **2**, 97–111 (1943)
80. Lynen, F., *Ann.*, **552**, 270–306 (1942); *Chem. Abstracts*, **37**, 5445 (1943)
81. Lynen, F., *Ann.*, **554**, 40–68 (1943)
82. Breusch, F. L., *Science*, **97**, 490–92 (1943)
83. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355–62 (1943)
84. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **150**, 413–25 (1943)
85. Westerfeld, W. W., and Berg, R. L., *J. Biol. Chem.*, **148**, 523–28 (1943)
86. Barnes, R. H., and Lerner, A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 216–19 (1943)
87. Keil, W., *Z. physiol. Chem.*, **274**, 175–85 (1942); *Chem. Abstracts*, **37**, 5943 (1943)
88. Keil, W., *Z. physiol. Chem.*, **276**, 26–32 (1942); *Chem. Abstracts*, **37**, 6728 (1943)
89. Artom, C., and Swanson, M., *J. Biol. Chem.*, **148**, 633–39 (1943)
90. Hansen, A. E., and Wiese, H. F., *Proc. Soc. Exptl. Biol. Med.*, **52**, 205–8 (1943)
91. Bailey, B. E., *J. Fisheries Research Board Can.*, **6**, 109–12 (1943); *Chem. Abstracts*, **37**, 6723 (1943)
92. Anthony, D. S., Quackenbush, F. W., Ihde, A., and Steenbock, H., *J. Nutrition*, **26**, 303–8 (1943)
93. Nitschke, E., *Ber. naturforsch. Ges. Freburg. Breisgan*, **37**, 43–64 (1941); *Chem. Abstracts*, **37**, 4433 (1943)
94. Boer, J., and Jansen, B. C. P., *Arch. neerland. physiol.*, **26**, 1–177 (1942); *Chem. Abstracts*, **37**, 4444 (1943)
95. Geyer, R. P., Boutwell, R. K., Elvehjem, C. A., and Hart, E. B., *Science*, **98**, 499 (1943)
96. Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *J. Dairy Sci.*, **26**, 429–37 (1943)
97. Deuel, H. J., Jr., Movitt, E., and Hallman, L. F., *Science*, **98**, 139–40 (1943)
98. Euler, B. V., Euler, H. V., and Säberg, I., *Ernährung*, **7**, No. 3, 65–74 (1942); *Chem. Abstracts*, **37**, 4443 (1943)
99. National Research Council, *Reprint Circ. Series*, no. **118**, p. 4 (August, 1943)

100. HEYWANG, B. W., *Poultry Sci.*, **21**, 521–24 (1942)
101. FRENCH, C. E., *J. Nutrition*, **23**, 375–84 (1942)
102. BURR, G. O., AND BARNES, R. H., *Ann. Rev. Biochem.*, **12**, 157–82 (1943)
103. BURR, G. O., AND BARNES, R. H., *Physiol. Revs.*, **23**, 256–78 (1943)
104. FRENCH, C. E., AND ELLIOTT, R. F., *J. Nutrition*, **25**, 17–21 (1943)
105. BUNKFELDT, R., AND STEENBOCK, H., *J. Nutrition*, **25**, 479–89 (1943)
106. REIFMAN, A. G., HALLMAN, L. F., AND DEUEL, H. J., JR., *J. Nutrition*, **26**, 33–42 (1943)
107. SLANETZ, C. A., AND SCHARF, A., *Proc. Soc. Exptl. Biol. Med.*, **53**, 17–19 (1943)
108. HOVE, E. L., *Science*, **98**, 433–34 (1943)
109. PAVCEK, P. L., AND SHULL, G. M., *J. Biol. Chem.*, **146**, 351–55 (1942)
110. STROHECKER, R., AND BUCHHOLZ, E., *Fette u. Seifen*, **49**, 351–53 (1942); *Chem. Abstracts*, **37**, 5763 (1943)
111. MILLER, H. G., *J. Nutrition*, **26**, 43–50 (1943)
112. CLAUSEN, D. F., BARNES, R. H., AND BURR, G. O., *Proc. Soc. Exptl. Biol. Med.*, **53**, 176–78 (1943)
113. GYÖRGY, P., AND TOMARELLI, R., *J. Biol. Chem.*, **147**, 515–24 (1943)
114. CLAUSEN, D. F., BARNES, R. H., AND BURR, G. O. (Unpublished results)
114a. LEA, C. H., "Rancidity in Edible Fats," *Dept. Sci. Ind. Research, Food Invest., Special Rept. No. 46*, p. 95 (London, 1938)
115. GYÖRGY, P., STILLER, E. T., WILLIAMSON, M. B., *Science*, **98**, 518–20 (1943)
116. BARNES, R. H., LUNDBERG, W. O., HANSON, H. T., AND BURR, G. O., *J. Biol. Chem.*, **149**, 313–22 (1943)
117. HOUCHIN, O. B., *J. Biol. Chem.*, **146**, 313–21 (1942)
118. VAN FLEET, D. S., *J. Am. Chem. Soc.*, **65**, 740 (1943)
119. SUMNER, R. J., AND TRESSLER, D. K., *Ind. Eng. Chem.*, **35**, 921 (1943)
120. AXELROD, B., AND KIES, M. W., *Federation Proc.*, **2**, 58 (1943)
121. BALLS, A. K., AXELROD, B., AND KIES, M. W., *J. Biol. Chem.*, **149**, 491–504 (1943)
122. DAM, H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 285–87 (1943)
123. GROLLMAN, A., AND HARRISON, T. R., *Proc. Soc. Exptl. Biol. Med.*, **52**, 162–65 (1943)
124. KAMEN, G. F., *Proc. Soc. Exptl. Biol. Med.*, **52**, 363–64 (1943)
125. ROFFO, A. H., AND ROFFO, A. E., JR., *Bol. inst. med. exptl. estud. cancer*, **19**, 503–30 (1942); *Chem. Abstracts*, **37**, 6335 (1943)
126. DICKENS, F., AND WEIL-MALHERBE, H., *Cancer Research*, **2**, 560–66 (1942)
127. MERELLI, E., *Tumori* (2), **14**, (26), 487–502 (1940); *Chem. Abstracts*, 37, 1767 (1943)
128. LISLE, E. B., *J. Soc. Chem. Ind.*, **61**, 148 (1942)
129. WICKS, L. F., AND SUNTZEFF, V., *J. Natl. Cancer Inst.*, **3**, 221–26 (1942)
130. WYNN, W., GIDDINGS, G., AND HALDI, J., *Federation Proc.*, **2**, 56 (1943)
131. LOEWY, A., FREEMAN, L. W., MARCHELLO, A., AND JOHNSON, V., *Am. J. Physiol.*, **138**, 230–35 (1943)
132. JOHNSON, V., LONGINI, J., AND FREEMAN, L. W., *Science*, **97**, 400 (1943)
133. LONGINI, J., AND JOHNSON, V., *Am. J. Physiol.*, **140**, 349–53 (1943)
134. JOSEPHS, H. W., HOLT, L. E., JR., TIDWELL, H. C., AND KAJDI, C., *Bull. Johns Hopkins Hosp.*, **71**, 84–95 (1942)

135. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, **147**, 371–78 (1943)
136. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405–14 (1943)
137. Burmaster, C. F., *Federation Proc.*, **2**, 59 (1943)
138. Woolley, D. W., *J. Biol. Chem.*, **147**, 581–91 (1943)
139. Artom, C., and Fishman, W. H., *Federation Proc.*, **2**, 1–2 (1943)
140. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 415–22 (1943)
141. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 423–30 (1943)
142. Cristol, P., Monnier, P., and Lazerges, P., *Trav. membres soc. chim. biol.*, **23**, 1078–85 (1941); *Chem. Abstracts*, **37**, 176 (1943)
143. Haven, F. L., and Levy, S. R., *Cancer Research*, **2**, 797–98 (1942)
144. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **150**, 47–55 (1943)
145. Zilversmit, D. B., Entenman, C., Fishler, M. C., and Chaikoff, I. L., *J. Gen. Physiol.*, **26**, 333–40 (1943)
146. Hunter, F. E., and Levy, S. R., *J. Biol. Chem.*, **146**, 577–81 (1942)
147. Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Welch, E. A., and Levy, S., *Federation Proc.*, **2**, 63 (1943)
148. Dimter, A., *Z. physiol. Chem.*, **270**, 247–65 (1941); *Chem. Abstracts*, **37**, 3167 (1943)
149. Dimter, A., *Z. physiol. Chem.*, **271**, 293–315 (1942)
150. Dimter, A., *Z. physiol. Chem.*, **272**, 189–200 (1942)
151. Schuwirth, K., *Z. physiol. Chem.*, **278**, 1–6 (1943); *Chem. Abstracts*, **37**, 5769 (1943)
152. Klenk, E., and Schumann, E., *Z. physiol. Chem.*, **272**, 177–88 (1942); *Chem. Abstracts*, **37**, 2762 (1943)
153. Klenk, E., and Rennkamp, F., *Z. physiol. Chem.*, **273**, 253–68 (1942); *Chem. Abstracts*, **37**, 5743 (1943)
154. Weil, A., and Liebert, E., *Quart. Bull. Northwestern Univ. Med. School*, **17**, 117–20 (1943)
155. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Biochem.*, **3**, 203–15 (1943)
156. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150**, 315–24 (1943)
157. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **149**, 505–9 (1943)
158. Bloch, K., Berg, B. N., and Rittenberg, D., *J. Biol. Chem.*, **149**, 511–17 (1943)
159. Rosenheim, O., and Webster, T. A., *Biochem. J.*, **37**, 513–14 (1943)
160. Rosenheim, O., and Webster, T. A., *Biochem. J.*, **37**, XIX (1943)
161. Heinrich, M. R., and Mattill, H. A., *Proc. Soc. Exptl., Biol. Med.*, **52**, 344–46 (1943)
162. Foldes, F. F., and Beecher, H. K., *J. Pharmacol.*, **78**, 276–81 (1943)
163. Glomset, D. A., and Bollman, J. L., *Gastroenterology*, **1**, 776–83 (1943)
164. Peters, J. P., and Man, E. B., *J. Clin. Investigation*, **22**, 707–14 (1943)
165. Peters, J. P., and Man, E. B., *J. Clin. Investigation*, **22**, 715–20 (1943)
166. Peters, J. P., and Man, E. B., *J. Clin. Investigation*, **22**, 721–26 (1943)
167. Thomas, E. M., *Am. J. Diseases Children*, **65**, 770–75 (1943)
168. Winkler, A. W., Durlacher, S. H., Hoff, H. E., and Man, E. B., *J. Exptl. Med.*, **77**, 473–86 (1943)
169. Kaplan, A., Entenman, C., and Chaikoff, I. L., *Endocrinology*, **32**, 247–58 (1943)

170. Josephs, H. W., *Am. J. Diseases Children*, **65**, 712–27 (1943)
171. LeWinn, E. B., and Zugerman, I., *J. Lab. Clin.* Med., **28**, 190–92 (1942)
172. Ravdin, I. S., Thorogood, E., Riegel, C., Peters, R., and Rhoads, J. E., *J. Am. Med. Assoc.*, **121**, 322–24 (1943)
173. Treadwell, C. R., Tidwell, H. C., and Grafa, B. G., Jr., *J. Biol. Chem.*, **149**, 209–15 (1943)
174. Deuel, H. J., Jr., and Davis, A., *J. Biol. Chem.*, **146**, 649–53 (1942)
175. Szego, C. M., and Barnes, R. H., *Endocrinology*, **32**, 367–68 (1943)
176. Chaikoff, I. L., Eichorn, K. B., Connor, C. L., and Entenman, C., *Am. J. Path.*, **19**, 9–22 (1943)
177. Blumberg, H., and Grady, H. G., *Arch. Path.*, **34**, 1035–41 (1942)
178. Newburgh, L. H., *Arch. Internal Med.*, **70**, 1033–96 (1942)
179. Hetherington, A. W., *Am. J. Physiol.*, **140**, 89–92 (1943)
180. Salcedo, J., Jr., Stetten, D., Jr., *J. Biol. Chem.*, **151**, 413–16 (1943)
181. Newburgh, L. H., *Ann. Internal Med.*, **17**, 935 (1942)
182. Tompkins, E. H., *Arch. Path.*, **35**, 695–712 (1943)
183. Tompkins, E. H., *Arch. Path.*, **35**, 787–802 (1943)
184. Bossa, G., *Rev. sud-americana endocrinol. immunol. quimioterap.*, **25**, 205–6 (1942)
185. Garb, J., *Ann. Internal Med.*, **19**, 241–52 (1943)
186. Krause, R. F., *J. Biol. Chem.*, **149**, 395–404 (1943)

Reviews

187. Newburgh, L. H., "Obesity," *Arch. Internal Med.*, **70**, 1033–96 (1942)
188. Bloor, W. R., "Biochemistry of the Fatty Acids and their Compounds, the Lipids," *Am. Chem. Soc., Series Technologic Monographs*, 387 pp. (Reinhold Publ. Corp., New York, 1943)
189. Burr, G. O., and Barnes, R. H., "Non-Caloric Functions of Dietary Fats," *Physiol. Revs.*, **23**, 256–78 (1943)
190. MacKay, E. M., "The Significance of Ketosis," *J. Clin. Endocrinol.*, **3**, 101–10 (1943)
191. Weinhouse, S., "The Blood Cholesterol," *Arch. Path.*, **35**, 438–500 (1943)
192. Hirsch, E. F., and Weinhouse, S., "The Role of the Lipids in Atherosclerosis," *Physiol. Revs.*, **23**, 185–202 (1943)
193. Ingle, D. J., "Relationship of the Adrenal Cortex to the Metabolism of Fat," *J. Clin. Endocrinol.*, **3**, 603–12 (1943)
194. Best, C. H., and Lucas, C. C., "Choline-Chemistry and Significance as a Dietary Factor," *Vitamins and Hormones*, **1**, 1–51 (1943)
195. Frame, E. G., "Lipotropic Substances," *Yale J. Biol. Med.*, **14**, 229–55 (1942)

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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In the compilation of this review an attempt has been made to give appropriate recognition to the current interest in normal and emergency nutrition, by discussing such aspects as the amino acid needs of the human subject, the synthesis of blood proteins, intravenous alimentation, and the use of urea by ruminants. References to papers in the fields of plant and bacterial metabolism have been largely omitted. During the year many delayed foreign journals have become available, but their arrival has been erratic and it has not been possible to fill some of the gaps. Throughout the review *l* and *d* have been used in the configurational sense.

Essential amino acids.—The amino acid requirements for maintenance in the adult human subject have recently been tested with protein hydrolyzates and with mixtures of purified amino acids as the chief or sole source of protein nitrogen. Studies of the former type have come chiefly from the laboratory of Holt. Nitrogen equilibrium was readily maintained on an enzymic hydrolyzate of casein, but this was not possible with acid-hydrolyzed casein until tryptophane had been added (1, 2). Two other acid hydrolyzates were prepared, one from casein which had been deaminized to destroy the lysine (3) and the other from casein which had been oxidized with hydrogen peroxide (4, 5) to convert the methionine to the corresponding biologically inactive sulfone (6) which was removed as the insoluble calcium sulfonate. From the latter preparation most of the residual cystine was also removed by adsorption on norit. When the former hydrolyzate was fed in diets fortified with tryptophane, equilibrium was not attained until lysine was added (7, 8). Supplementation of the latter hydrolyzate with tryptophane, methionine, and cystine provided a mixture adequate for maintenance. Removal of the methionine produced negative nitrogen balances and loss of body weight; its restoration re-established nitrogen equilibrium in a few days, but did not increase the body weight. The inconsistent results obtained by the omission of cystine were considered to suggest that cystine was essential, but this conclusion was thought to require confirmation (9). An abstract

from the same laboratory indicates that a diet deficient in arginine maintained nitrogen equilibrium for a period of ten days but gives no preparative details (10). The tryptophane-deficient regimen was tolerated for at least five weeks, with no untoward effect (1), but the lysine-deficient diet produced nausea, dizziness, and a hypersensitivity to metallic sounds presumed attributable to the sharp rise in excretion of non-keto organic acids during the deficiency period (8). Possibly the excretion of such acids does constitute a "biochemical lesion characteristic of lysine deficiency" (8), but one is inclined to wonder whether the acid residues produced in the deamination of the casein were not a contributing cause of considerable importance in these particular tests. On the ninth day of the arginine-deficient diet, the seminal plasma of the three male subjects showed only a tenth of the normal number of spermatozoa; the authors therefore consider arginine an essential amino acid and assume that nitrogen equilibrium was maintained during the ten-day period because atrophy of the spermatogenic tissue provided the arginine required. This intriguing possibility deserves further study.

Results of tests in the human subject fed highly purified amino acids have been announced by Rose and his associates in two short notes (11, 12). Nitrogen equilibrium was readily maintained when over 95 per cent of the nitrogen was fed in the form of the ten essential amino acids, the nine previously shown to be indispensable for maintenance in the rat (13) and the adult dog (14), and the tenth (arginine) required for maximum weight increment in the young rat (13). Removal of valine (11) or of isoleucine or phenylalanine (12) from the mixture caused a more marked negative nitrogen balance than did the removal of methionine (11) or of threonine or leucine (12); in each case addition of the missing amino acid promptly restored equilibrium. Curiously, histidine was not required for the maintenance of nitrogen equilibrium, or even for its restoration after a period of marked negative balance induced by the simultaneous removal of isoleucine or phenylalanine (12). Subsequent tests have indicated that tryptophane and lysine are also essential for maintenance, but that arginine is not (15). Publication of the details of these observations and the outcome of quantitative studies now in progress will be awaited with considerable interest.

Dogs starved for several days can be kept in nitrogen equilibrium when supplied their non-protein needs by gavage and given mixtures of the nine amino acids essential for maintenance by intravenous

injection. Mixtures which contained glycine instead of histidine supported positive balances for the first two or three days, after which nitrogen excretion increased and the balances became negative. In contrast, the administration of a tryptophane-deficient hydrolyzate of casein was always followed by a markedly negative balance, even on the first day (16).

The nine amino acids required for growth in the rat appear to be indispensable also in the mouse (17). Since removal of arginine from the dietary mixture of twenty purified amino acids did not retard growth, the capacity of the mouse to synthesize arginine was probably appreciable; inasmuch, however, as weight increments on the complete mixtures were sub-maximal, the evidence was inadequate to establish dispensability for maximum growth. Tests showed that a mixture of arginine and the nine essential amino acids supported slow growth; hence none of the ten other amino acids omitted could have been absolutely indispensable.

Regeneration of hemoglobin and blood plasma and tissue proteins.—The war has emphasized the problem of shock and stimulated the study of such biochemically related aspects as the regeneration of hemoglobin and plasma proteins and the repair of body tissues. Only a small portion of this literature can be considered here.

Direct evidence of interchange between plasma and tissue proteins (18) has been sought by transfusion and perfusion tests. Repeated transfusion into cats of fluid which contained plasma proteins in smaller concentrations than were present in the blood increased both the protein content and the volume of the blood plasma, probably because the animal's storage depots became saturated and could no longer take up protein from the transfusing fluid. Perfusion of surviving livers, with fluid which contained plasma protein and with fluid devoid of protein, suggested that the direction of movement depended upon the liver reserves and the protein content of the fluid. Transfusion into hepatectomized animals seemed to indicate that more plasma protein could leave the blood than was necessary for normal metabolism, and conversely, that it could arise from some other source than the liver (19). Immediately after complete removal of the liver from the dog a small loss (or dilution) of total plasma protein occurred. There was a similar change in albumin content, a marked loss of fibrinogen and euglobulin, but an increase in pseudoglobulin. Little further change was noted. After plasmapharesis no regeneration of plasma protein was observed in nine hours (20).

In dogs maintained on low protein diets the hyperproteinemia produced by repeated injections of homologous plasma was greater than in dogs fed high protein diets, but the albumin-globulin ratio was lower (21). The hypoalbuminemia produced in dogs by three weeks of dietary protein deprivation could be corrected by the transfusions of large volumes of plasma daily (50 cc. per kg.) for a week, but during the following two weeks the plasma proteins returned to their previous low levels and the output of urinary nitrogen became greater than before (22). It was therefore considered probable that the capacity to convert plasma proteins into proteins of other tissue is limited enough to make small repeated injections more effective (23) than a single massive dose.

Evidence that surgical shock may be a manifestation of hypoalbuminemia amenable to correction by the administration of amino acids has been reviewed at intervals by Elman (24) who for some time has championed the use of protein hydrolyzates as a non-antigenic source of protein nitrogen for safe parenteral administration. In dogs with hypoalbuminemia of dietary origin, the continuous intravenous injection over a 48-hour period of a pancreatic hydrolyzate of casein which contained 3.5 gm. of nitrogen produced a positive nitrogen balance and a regeneration of serum albumin which persisted for at least a week; when the period of injection was shortened to 24 hours, proportionately less nitrogen was retained and the increase in serum albumin was not maintained (25). The mean survival time of dogs bled 10 cc. per kg. hourly was 3.7 hours. Injection with a pancreatic digest of casein increased the survival time to 5.15 hours, with a mixture of the ten essential amino acids to 4.25 hours; an intermediate increase was noted with serum and with citrated plasma, but none at all with glucose (26).

A mixture of the ten purified amino acids essential for maximum growth in the rat (13) was as effective by mouth as most native proteins for the production of plasma protein in standardized dogs in which depletion had been effected (27) by the removal of blood and the return of the washed cells (plasmapharesis). Most of the mixtures contained also glycine, to the extent of about one-fourth by weight. The 2.3 to 2.7 gm. of nitrogen supplied per day were adequate to maintain health and nitrogen equilibrium over test periods as long as six weeks, and were better tolerated by vein than an equivalent amount of any protein digest tested. When the nitrogen allotment was reduced by omission of the glycine, the nitrogen balance remained posi-

tive and good plasma protein synthesis continued, but there was a small loss in body weight; an attempt to compensate for the glycine omission by increasing the proportions of several of the essential amino acids in the mixture did not improve the utilization. Removal of arginine for a week or two had no effect on maintenance, but did allow a slow fall in the output of plasma protein which returned gradually to normal when the arginine was restored. The simultaneous omission for one week of arginine, histidine, and most of the lysine from a mixture which contained also cystine, glycine, alanine, tyrosine, and glutamic acid, caused a marked drop in the production of plasma protein, but supported body weight and nitrogen equilibrium. Under conditions not definitely determined, cystine (but not methionine) may stimulate the production of plasma protein for a week or more; apparently its capacity to do this when added to a protein-free, but otherwise adequate diet, depends partly upon the nature of the protein fed previously. On an amino acid mixture in which methionine was replaced by cystine, production of plasma protein continued for a similar period, but dogs lost weight and showed a negative nitrogen balance. Withdrawal of valine or threonine from the mixtures was followed both by a sharp decline in the production of plasma protein and by a negative nitrogen balance.

The regeneration simultaneously of both hemoglobin and plasma protein in dogs fed low protein diets with abundant iron, but bled to sustain anemia, has been similarly studied (28). Invariably the dogs produced more hemoglobin than plasma protein, usually 1.5 to 4 times as much, even when they were given digests of dog plasma or serum intravenously; dog or sheep hemoglobin injected intraperitoneally was remarkably well utilized to form both. Mixtures of the purified amino acids necessary for maximum growth in the rat, plus glycine, were as effective for total protein production as digests of hemoglobin, serum, or casein, whether given by vein or by mouth. They favored hemoglobin production even more than did digests of hemoglobin or hemoglobin itself, so much so that the ratio of hemoglobin to plasma protein became too high to allow the hemoglobin level to be lowered adequately by bleeding, without simultaneously producing a dangerous hypoproteinemia. Casein digests favored plasma protein production to about the same extent as serum digests. Replacement of the methionine in the amino acid mixture by cystine allowed the production of hemoglobin and plasma protein to continue, but yielded proportionately more plasma protein than had the original mixture. Ap-

parently cystine and choline can together induce the formation of both hemoglobin and plasma protein.

Dogs made hypoproteinemic by low-protein intake and plasmapharesis incorporated more radioactive sulfur into the plasma protein when the sulfur was fed in the form of cystine than when it was fed as methionine or homocystine plus betaine (29). Upon isolation and intravenous injection, plasma protein which contained radioactive sulfur disappeared at the same rate from the blood of dogs in hemorrhagic shock as from normal animals (30).

In normal fasted rats from which blood equivalent to 3.5 to 4.2 per cent of the body weight was removed slowly to produce shock, the whole blood and plasma amino acid levels rose rapidly, but only after the blood pressure had fallen between 85 and 90 mm. Hg. Correlation with other observations suggested that anoxia caused by decreased blood supply had lowered the capacity of the liver to effect deamination and had accelerated the breakdown of protein in the peripheral tissues (31). In dogs, the rapidity with which plasma proteins were regenerated in the first 72 hours following a single severe non-fatal hemorrhage was not affected by alimentation (32). Rats with hepatitis induced by restriction of diet and exposure to carbon tetrachloride showed a hypoproteinemia attributable almost entirely to loss of albumin; the limited functional capacity of the liver prevented the type of protein fed from exercising its usual influence upon the plasma protein level (33). Dogs fed low-protein diets for several weeks proved to be very susceptible to chloroform injury of the liver, but methionine afforded protection when it was given just before or within four hours after the anaesthesia. Choline plus cystine (but not choline alone) showed a similar effect. The results were ascribed to reincorporation of sulfur into the protein matrix of the liver from which it had been lost during the period of protein depletion; possibly it is required for the proper functioning of vital enzyme systems (34). Electrophoretic analyses have confirmed the view that the changes in serum protein associated with hepatic insufficiency are due in part to impaired albumin production; the most consistent and characteristic abnormality noted, however, was the increase in the γ-globulin fraction, which has the highest molecular weight (35).

Numerous tests have been made of the suitability of gelatin as a colloidal and non-antigenic emergency substitute for plasma. After its injection intravenously into protein-depleted animals some excretion into the urine occurred, but evidence was also obtained for its

partial retention, probably unchanged, for 24 hours and for its partial metabolism (36). In normal dogs half of the gelatin injected was lost in half an hour, but some remained even after three days. Only a third or less of the amount lost from the plasma was recoverable from the urine (37). In dogs subjected to hemorrhagic or burn shock, gelatin infusion was superior to saline; again, not all of the gelatin lost from the blood could be recovered in the urine (38).

Utilization of non-protein nitrogen in the ruminant.—Evidence for the synthesis of protein in the rumen from non-protein nitrogenous source has recently been reviewed by Goss (39). The few studies mentioned here contain data of supplementary interest. Calves were unable to maintain nitrogen balance on diets which contained 4.4 per cent of protein, but grew satisfactorily when enough urea was added to make the nitrogen content of the diet equivalent to 16.2 per cent of protein; on the diet thus fortified the digestibility of dry matter and carbohydrates was apparently better, a fourth to a third of the nitrogen was retained, and the muscle tissue produced was normal in crude protein content (40). When 25 per cent of the total nitrogen intake of dairy cows was as urea, milk yields and body weights were usually well maintained and the percentages of protein, fat, lactose, and total solids in the milk were not altered. The urea content of the milk approximated that of the blood and never exceeded 28 mg. per 100 cc. The urea wastage, which always occurred, was less after a preliminary period on a diet deficient in protein (41). *In vitro* tests of the liquor from a steer with a rumen fistula suggested that microorganisms in the paunch produce ample amounts of urease to convert rapidly to ammonia all of the urea likely to be fed (42). Incubation of the rumen liquor under various conditions indicated that both protein synthesis and protein breakdown may occur. Starch was especially effective in promoting synthesis, gelatin in inducing hydrolysis. Hence the feeding of non-protein nitrogen probably favors the synthesis of protein when the starch content of the diet is sufficiently high to balance the nitrogen present; under certain conditions it may diminish protein breakdown. The biological value of a protein for ruminants may therefore depend not only on its amino acid content, but also on whether its presence in the paunch promotes protein synthesis or protein hydrolysis (43).

d-Amino acids.—A comprehensive review of the metabolism of the *d*-amino acids may be found elsewhere (44). Recent tests with a strain of *Bacillus subtilis* have revealed the excretion of a peptide com-

posed of *d*-glutamic acid units linked through the ω-carboxyl groups and showing no response to the biuret test and no susceptibility to racemization (45). Hydrolysis of gramicidin by a procedure known to cause slight racemization produced leucine which was largely of the *d* configuration (46), thus confirming previous observations. The valine was racemic, either because both enantiomorphs coexist in gramicidin or because the valine was selectively racemized during the hydrolysis (46, 47). The first alternative is indicated by the intermediate isolation from partially hydrolyzed gramicidin of an optically inactive benzoyl valylvaline (48). The phenylalanine from tyrocidine was predominantly of the *d* configuration; the other amino acids were mainly *l* (49).

The possible existence of *d*-glutamic acid in tumor tissue is the subject of several German papers which have become available in the past year. Abderhalden has discussed the problem of avoiding contamination with inorganic salts and other amino acids in the usual methods of isolating glutamic acid; from studies in which he has effected purification through the ester he concludes that certain types of carcinoma tissue contain no *d*-glutamic acid; that tumor tissue may yield it, but in much smaller amount than claimed by Kögl; and that its presence is probably a reflection of failure of enzyme systems to function normally (50, 51). Klingmüller has studied several isolation procedures critically and found them to yield too little of the *d* acid to warrant considering it a significant component of malignant tissue (52). Wieland has employed a new isolation procedure involving selective adsorption of the dicarboxylic acids from acidic solution; removal of adsorbed cystine by simultaneous reduction and elution with a saturated solution of hydrogen sulfide; and final elution with barium hydroxide solution to provide an eluate from which pure glutamic acid hydrochloride could readily be prepared for optical activity determination. Although 80 per cent of the total glutamic acid and 71 per cent of the admixed *d* acid were recovered consistently from test solutions, more than 2 per cent of the *d* isomer was never found in the glutamic acid isolated from the hydrolyzates of several types of tumor tissue (53). On the other hand, Kögl, Erxleben & van Veersen have reexamined the question, using *dl*-, *d*- and *l*-glutamic acid marked with deuterium to aid in estimating the completeness of isolation. Their data indicated degrees of racemization lower than originally reported, but ranging from 3 to 4 per cent for some types of tissue up to 40 or 48 per cent for others. The authors empha-

size the consistency with which certain types of tissue have yielded small amounts of *d* acids, others large amounts, in their several years of experience. They also observe that they have obtained higher yields of glutamic acid than have other analysts (54). Unfortunately neither argument is valid, *per se*.

Abderhalden has suggested that *dl*-amino acids are produced *in vivo* by the amination of α-keto acids, but that appreciable accumulation of the *d* component is normally prevented by the *d*-amino acid oxidase system; hence, that the contributing cause for the existence of racemic amino acids in tumor tissue may be the destruction of this enzyme system and a consequent availability for protein synthesis of abnormally high concentrations of racemic amino acids (50). The validity of the basic assumption is open to question, but in certain malignancies the *d*-amino acid oxidase system does seem to be affected. A statistically significant depression from normal *d*-amino acid oxidase activity was observed in extracts of the livers and kidneys of rats with Walker carcinomas (55). The riboflavin content of the livers was normal (56), and the *d*-amino acid oxidase activity could not be accelerated by addition of excess flavin-adenine-dinucleotide. Calculations showed that the specific protein component was deficient both in the liver and the kidney (57). Complete removal of the carcinoma was followed by a return to a normal liver content of *d*-amino acid oxidase in ten or twelve days (58). Shack has also observed reduction of *d*-amino acid oxidase activity in the liver of tumor-bearing rats (59).

The *d* modifications of methionine and phenylalanine were available to the mouse for growth, but the *d* isomers of valine, leucine, isoleucine, and threonine were not (17). Isotopic *d*-lysine with N^{15} in the α-amino group and deuterium in the β, γ, and δ positions was added in small amounts daily for four days to the stock diet fed growing rats. About half of the amount ingested was excreted unchanged; some deamination of the balance occurred, but the *l*-lysine isolated from the tissue proteins contained neither deuterium nor heavy nitrogen (60).

Albanese & Irby have cautioned against the use of racemic amino acids in essential amino acid mixtures, on the ground that the unutilizable optical isomers may exert a toxic influence (61). The assumption is attractive, but unfortunately quite unwarranted by the evidence presented. The only isoleucine in the essential amino acid mixture used was part of an "*l*-leucine-isoleucine mixture" of unstated com-

position. The marked loss in weight of all rats fed the essential amino acid mixture is in striking contrast with the experiences of others (13, 17, 27) with essential acid mixtures which contained racemic amino acids in similar proportions. The possible existence of an overshadowing deficiency in the diet was not investigated, nor was any attempt made to verify or account for the reported nitrogen retentions of 0.154 to 0.381 gm. per rat in a twelve-day period during which 10 to 21 gm. of the initial 44 to 61 gm. of body weight were lost.

Deamination.—*l*-Amino oxidase has been isolated from the kidney of the rat, rabbit, cat, mouse, and pig and from the liver of the rat, by low temperature acetone precipitation and salt fractionation. The activity of the contaminating *d*-amino acid oxidase was eliminated by precipitating its protein component with the *l*-amino acid oxidase under conditions which did not also precipitate the flavin component. The *l* enzyme reacts with molecular oxygen and hydrogen acceptors, such as methylene blue, and is insensitive to caprylic alcohol and cyanides. During anaerobic oxidation in the presence of catalase a molecule each of ammonia and the keto acid was produced for each atom of oxygen absorbed; in the absence of catalase, hydrogen peroxide accumulated and the ratio of oxygen to ammonia approximated 2 to 1. The oxidase had little or no effect on aspartic acid, glutamic acid, arginine, lysine, ornithine, serine, or threonine and no effect on β-alanine or the *d*-amino acids. Of the twelve amino acids affected, leucine was the most readily oxidized, histidine and alanine were the least. Keto acids corresponding to leucine, isoleucine, and methionine were isolated (62).

Resting suspensions of *B. coli* and other bacterial species deaminated serine both aerobically and anaerobically to produce pyruvic acid, but the deamination was usually effectively blocked by esterification of the hydroxyl group. Threonine was converted anaerobically to α-ketobutyric acid, but alanine was not attacked. In the presence of magnesium chloride, cell-free liver extracts deaminated serine anaerobically in a similar manner. The mechanism probably involved removal of the elements of water to form the α, β-unsaturated α-amino acid, followed by rearrangement to the α-imino acid and hydrolysis of the latter to yield the corresponding α-keto acid and ammonia (63).

Urea formation.—In the past year Krebs has published a critique of experimental findings which have been interpreted as at variance with the ornithine cycle theory (64). Leuthardt's observation that the liver of starved guinea pigs forms urea more rapidly from gluta-

mine than from ammonium chloride (65) was verified experimentally, but the need of assuming a more direct conversion of the amide nitrogen of glutamine to urea than via ammonium glutamate and the ornithine cycle was challenged by demonstrating that ammonium glutamate was converted to urea quite as rapidly as glutamine. The report of Borsook & Dubnoff that rapid synthesis of arginine is effected by the kidney cortex from citrulline and glutamic acid (66) was also confirmed; tests based on acceleration of urea production by glutamic acid did not warrant assuming that an analogous reaction occurred in the liver. The increased rate of urea production reported by Bach (67) from citrulline or ornithine in the presence of α-ketoglutarate or pyruvate was not confirmed. The results of the perfusion studies with rat liver which Trowell interpreted as contradicting the ornithine cycle concept (68) were attributed, for the most part, to his use of unfavorable experimental conditions.

Evidence submitted by Gornall & Hunter (69) has lent considerable support to the ornithine cycle theory. In the presence of ammonia, carbon dioxide, and lactate, ornithine and citrulline showed approximately the same capacity to stimulate the synthesis of urea by liver slices from starved rats, and the effect of the two together never exceeded the maximal effect of one alone. Measurement with a suitable colorimetric method (70) showed that citrulline synthesis accompanied the accelerated urea production induced by adding ornithine. Thus, the speed of the over-all reaction in the cycle appears to be limited by the rate at which citrulline can be converted to arginine. Bach's ratio of one between ammonia nitrogen used up and urea nitrogen produced with citrulline as a catalyst (67) was fully confirmed, but was interpreted as in harmony with the theory of a continuously operating cycle, rather than as affording evidence, as Bach implied, that synthesis of urea from citrulline may not proceed via arginine. Gornall & Hunter point out that the ratio of 0.5 proposed for the synthesis by Krebs & Henseleit was based on a single experiment in which a ratio of 0.69 was actually found (71) and could apply only if the ornithine liberated with the urea were prevented from reentering the cycle. Ratios above one were found when large amounts of ornithine were provided, and were accounted for by the formation and accumulation of citrulline; contrary to the assumptions of Borsook & Dubnoff (66), this suggests that the kidney mechanism may actually serve to convert citrulline provided by the liver to arginine. The observations in the paper were in complete contrast with those of Trowell (68).

Ammonia production.—Attention has recently been called to the existence of a glutamine-like substance in blood plasma (72, 73), spinal fluid, and their ultrafiltrates (73). In dogs with explanted kidneys the amide nitrogen removed from the blood plasma was sufficient to account for the amide nitrogen eliminated as such in the urine, the ammonia removed from the kidney by the renal vein, and 60 per cent or more of the ammonia excreted in the urine (74). In hydrochloric acid acidosis the administration of glutamine increased the ammonia excretion; in alkalosis less amide nitrogen was removed. Blood urea was not used for ammonia production. In experiments in which the amide nitrogen did not account for all of the urinary ammonia, the α-amino nitrogen made up the difference. In a sense, these experiments represent an interesting modification of the theory proposed by Bliss (75, 76) some years ago. The existence of a glutaminase in the kidney has been known for several years (75, 77) and the existence of glutamine in animal tissues has been established (78, 73).

Transmethylation.—In the synthesis of creatine from guanidoacetic acid by rat liver slices, *d*-methionine was only half as effective as its natural antipode. The α-keto analogue was equally effective, but whether it was first converted into the *l*-amino acid was not determined. Benzoic acid, which inhibits *d*-amino acid oxidase, inhibited transmethylation by *d*-, but not by *l*-methionine. Since the methyl sulfonium chloride derivative was active, but the sulfoxide and the sulfone were not, oxidation of the sulfur atom was probably not involved in the process (79). Deuterium fed to a human subject for three days in the methyl group of methionine could be isolated, in larger percentages, from either the methyl group of the creatinine excreted in the urine or the methyl groups of choline prepared from the blood, than from either parent compound as a whole (80). Apparently transmethylation from choline to methionine is automatic and continuous. In rats fed deuteriocholine and homocystine the deuteriomethyl group was found in tissue methionine, but this was true also when the deuteriocholine was fed without the homocystine, or with ordinary methionine (81).

That anserine, the methyl derivative of carnosine, may derive its methyl group from "labile" methyl groups of the diet was shown in the rabbit fed deuteriomethionine. The deuterium predominated in the methyl groups of the anserine, choline, and creatine isolated from the tissues, and in the methyl groups of the creatinine separated from the urine (82).

Dosage with nicotinamide is followed by the urinary excretion of its methylated derivatives, trigonelline and N^1-methylnicotinamide (83). One per cent of nicotinamide fed in a 10 per cent casein diet inhibited growth in rats, apparently because it depleted the animals' supply of methyl groups. Growth was restored by the administration of methionine or of choline plus homocystine, but not by betaine or choline, or by cystine or homocystine. The unavailability of the methyl groups of betaine and choline was in contrast with their availability in other processes of methylation (84). The mechanism operates aerobically in liver slices (85).

Methionine and cystine.—Contrary to a previous report (86) mice grew well on diets which contained methionine, but only traces of cystine (87). Mesolanthionine (which lacks one of the sulfur atoms found in mesocystine) failed to promote growth in rats fed a cystine-deficient diet, probably because it was not split or because it yielded *d*-cysteine, which is not convertible to the *l* enantiomorph (88). The assumption that bromobenzene inhibits growth in rats by creating a cystine deficiency has been verified by correlating rate of growth with the cysteine excreted as bromphenylmercapturic acid and the organic sulfur available for growth (89). Phenyl-*l*-cysteine is acetylated by the rat and excreted as *l*-phenylmercapturic acid, as might have been assumed, though not heretofore proved (90). Diets high in *dl*-methionine (6.4 to 12.5 per cent) caused the liver to atrophy, but produced no lesions; food intake was poor, but the atrophy was not attributed to starvation. Cysteic acid (12.5 to 15 per cent of the diet) induced portal necrosis and cirrhosis of the liver, but taurine (1 to 10 per cent) produced no lesions (91).

Cystinuric dogs maintained on 10 and 25 per cent arachin diets produced much more "extra" urinary cystine from 2 gm. of cystine than from 2 gm. of methionine, the reverse of observations made earlier with casein (92). In the same dogs maintained on 10 per cent casein diets, cystine or cysteine derivatives in which either or both the sulfur and nitrogen groups were bound, yielded little or no extra urinary cystine; glycylcysteine gave less than a fifth, and homocysteine only a fourth as much extra cystine as an equivalent amount of cysteine. Neither homocysteine nor homocystine was excreted unchanged (93).

When methionine was incubated with liver brei it retained its sulfur, but underwent deamination; with liver slices a loss of both sulfur and nitrogen occurred and small amounts of cysteine were

produced, possibly from the tissue. Less cysteine was formed from homocystine and choline and none at all from the keto acid corresponding to methionine (94). The enzyme which produces hydrogen sulfide, ammonia, and pyruvic acid from cysteine has been prepared in partially purified form from both rat (95) and mouse (96) liver. It is similar to, if not identical with, the dehydrase which produces pyruvic acid from serine (96, 63). Since preparations from the liver, kidney, and pancreas show disproportionate production of hydrogen sulfide from cysteine and homocysteine, the desulfurase which attacks homocysteine is presumably not the same as the one which attacks cysteine (97). Incidentally, the addition of sodium sulfide to liver and kidney tissue did not inhibit oxygen consumption as it did with brain tissue (98).

The anaerobic decarboxylation of *l*-cysteic acid to taurine shown with liver extracts (99) can also be effected by kidney brei. In the presence of oxygen, kidney slices and kidney brei produced similar decreases of carboxyl groups, but the brei also caused deamination, as did intestinal mucosa (100).

Phenylalanine and tyrosine.—Contrary to earlier reports that configuration is without influence in human alcaptonuria, less than half as much homogentisic acid was excreted after the ingestion of 5 gm. of *d*-phenylalanine as after the ingestion of 5 gm. of the *l* form. Similar conclusions were reached with *l*- and *dl*-tyrosine. Phenylpyruvic acid was produced from the *d*-phenylalanine (101). Pregnancy was without influence (102).

In mice which had been fed phenylalanine or tyrosine for several weeks, a transitory "alkali" test for alcaptonuria could be obtained after a minimum oral intake of 0.25 gm. of *l*-phenylalanine or *l*-tyrosine; 0.25 gm. of *d*-phenylalanine and 0.13 gm. of *dl*-tyrosine were ineffective. Without the preliminary feeding period even massive doses of the natural isomers failed to produce the test. The urine gave a positive ferric chloride test, indicative of phenylpyruvic acid, after feeding either isomer of phenylalanine; and a positive Millon's test after the ingestion of *dl*-tyrosine (103).

Homogentisic acid has been isolated from the urine of rats fed 0.4 gm. of *l*-tyrosine per 100 gm. daily, after preliminary tyrosine feeding (104) and from the urine of rats fed diets containing 12 per cent of *l*-tyrosine for several days (105). *d*-Phenylalanine and *dl*-tyrosine in even larger doses produced too little homogentisic acid for detection by the "alkali" test (104). That small single parenteral

dosages of *l*-phenylalanine will produce alcaptonuria in rats (106) has been denied and attributed to reliance upon the ammoniacal silver reduction test which also responds to ketonic acids (104). Homogentisic acid exhibits chemiluminescence in alkaline solution (107) and may thereby be distinguished qualitatively from gentisic acid and other substances which do not (105).

Several recent papers (108, 109, 110) represent a reinvestigation of the syndrome which may occur in rats fed diets which contain 5 to 10 per cent of tyrosine (111, 112). The authors suggest tentatively that tyramine produced by the kidney may be the responsible agent (108, 109). Why homogentisic acid production should not occur in these rats (109) as in others (105) is not clear.

In the guinea pig fed extra tyrosine with a vitamin C–deficient diet, the administration of a small single dose of glutamic acid or other acid substance lowered the output of tyrosine metabolites, probably because it mobilized ascorbic acid from stores not otherwise called upon; repetition failed to produce the same effect, and change to a more alkaline state increased the output. Ascorbic acid fed with sodium bicarbonate did not induce the same lowering as ascorbic acid alone (113).

When premature and full term infants were maintained on a vitamin C–deficient diet calculated to furnish them 0.5 gm. of tyrosine and phenylalanine daily per kg. of body weight, and were fed 1 gm. of *dl*-phenylalanine in addition per kg. per day for several days, they excreted phenylalanine, enough tyrosine to crystallize from the urine, and large amounts of *p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids. No homogentisic acid was found, and the output of phenylpyruvic acid was extremely small, even after larger repeated dosages of phenylalanine. Ingestion of tyrosine produced the corresponding hydroxy and keto derivatives in large amount, but no phenylalanine. Vitamin C, given simultaneously with large repeated doses of phenylalanine or tyrosine, had little effect on the metabolite excretion (114). In a phenylpyruvic oligophrenic, phenylalanine, phenyllactic and phenylpyruvic acids were always excreted in the urine. The output could readily be increased by feeding phenylalanine, but not by feeding tyrosine. There was no evidence either of conversion of phenylalanine to tyrosine or of abnormal tyrosine metabolism. Large dosages of vitamin C did not affect the disturbance (115).

Further confirmation is had of an enzyme, extractable from the liver, which oxidizes *l*-phenylalanine without splitting off ammonia;

the extract acted similarly on *l*-tyrosine. A corresponding enzyme could not be isolated from the kidney (116).

Diiodotyrosine and thyroxine.—Conversion of radioactive iodide to diiodotyrosine and thyroxine occurred upon incubation with thyroid tissue slices in bicarbonate-Ringer's solution; homogenization or desiccation of the tissue prevented the change (117). The process was retarded by sulfanilamide (118) and by poisons known to inhibit aerobic oxidations involving the cytochrome oxidase system (119).

The thyroid activity produced by iodinating casein and soybean protein in bicarbonate solution reached a maximum when four atoms of iodine were added per molecule of tyrosine, enough for the substitution of two atoms in each molecule (120). Hydrolyzates of such iodinated casein prepared with barium hydroxide yielded 0.4 per cent of crystalline *dl*-thyroxine, 29 per cent of the total indicated by biological assay (121); hydrolysis with a sulfuric acid–butyl alcohol mixture allowed the isolation of 0.1 per cent of crystalline *l*-thyroxine (122). Comparisons indicated that the thyroid activity of iodinated casein differed in several respects from that of thyroglobulin (123).

Radioactive iodide injected intraperitoneally into rats deprived of all thyroid tissue was recoverable in appreciable proportions from the liver and intestines as diiodotyrosine and thyroxine (124).

Tryptophane.—The mode of development and type of cataract formed in rats fed diets deficient in tryptophane differed from those of cataracts associated with riboflavin deficiency or the feeding of galactose or xylose (125, 126). N-Methyltryptophane (*l*-abrine) prevented the cataract development and promoted growth, but in rats fed a diet deficient in "labile" methyl groups it did not prevent the accumulation of liver fat (127).

After fifty or more days, depending on age, rats fed tryptophane-deficient diets showed a reduction in plasma protein and hemoglobin, but not in erythrocyte count; though fed in amounts barely adequate for maintenance, similar diets supplemented with tryptophane prevented such change (128). Similar hemoglobin reduction was noted in young rats fed diets which contained only 3.5 per cent of lactalbumin, in contrast with controls given the same vitamin, mineral, and caloric allotments, but more lactalbumin (18 per cent); increased caloric and iron intakes afforded no improvement on the low-protein diet (129). To what extent the latter results may be attributable to differences in intake of tryptophane can at present only be surmised.

A short note indicates that female rats placed on a tryptophane-

deficient diet after insemination failed to cast litters (130), thus confirming a similar observation made accidentally several years ago (131). However, such an observation alone affords little justification for attributing (130) poor reproduction on low protein diets specifically to an inadequate intake of tryptophane; there is at present no basis for assuming that normal reproduction can occur on any diet markedly deficient in any of the several amino acids essential for growth.

Recent evidence indicates that the formula originally assigned to kynurenine (132) is incorrect. The new structure proposed is that of *o*-aminophenacylaminoacetic acid,

$$o\text{-}H_2N \cdot C_6H_4 \cdot CO \cdot CH_2 \cdot CH(NH_2) \cdot COOH,$$

the *dl* modification of which has been synthesized and compared with kynurenine isolated from the urine of rabbits fed tryptophane (133). The sulfates of the two products showed melting points which agreed well, they gave identical ultraviolet absorption spectra, showed identical titrations with alkali in alcoholic solution, and responded similarly to the tests for kynurenine outlined by Kotake (134). Both substances produced *o*-aminoacetophenone on heating with barium hydroxide and both stimulated the development of eye pigment in *v-Drosophila* and *a-Ephestia* (133).

Unfortunately it has been impossible to secure for appropriate review here several pertinent articles by Kotake, *et al.,* outlined in abstracts prepared abroad (134).

The green pigment-producing compound observed in the urine of pyridoxine-deficient rats (135) and pigs (136) has now been proved identical with xanthurenic acid (137), 4,8-dihydroxy-2-quinoline carboxylic acid (138). A few years ago this product was found to be excreted by rats and rabbits, but not in appreciable amount by dogs, fed high protein diets (139). It was produced in rats, rabbits, and guinea pigs fed large amounts of tryptophane, apparently being formed via kynurenine, but not via kynurenic acid (140), with which it may be coprecipitated (139). The impression should not be left that kynurenine and kynurenic acid are excreted only in the vitamin-deficient animal (137). This has not previously been intimated for kynurenic acid production; it has been suggested, but not definitely proved, for the production of kynurenine. The possible formation of even other quinoline derivatives (141) and of anthranilic acid (134) complicates the picture of tryptophane metabolism considerably,

but may help explain why such a small part of tryptophane or of kynurenine appears in the urine as kynurenine or kynurenic acid (142).

Histidine.—Several papers concerning the role of urocanic acid in histidine metabolism have only recently become available (143, 144, 145). As in most earlier studies, no urocanic acid was excreted after histidine was fed to dogs or injected in large doses into rabbits (143) or guinea pigs (145). Contrary to the report (146) that urocanic acid is formed as an intermediate by histidase none could be detected upon incubating histidine with liver tissue (143). However, aqueous liver extracts converted urocanic acid to a substance from which one equivalent of ammonia was liberated by strong alkali, but only a trace by sodium carbonate (143). The responsible enzyme (urocanicase) and histidase were each nearly completely separable from the other. The action of the histidase on histidine was depressed by the addition of urocanic acid (144), *d*-histidine, or pyruvic acid (143), but the action of urocanicase on urocanic acid was unaffected by the addition of either *l*- (144) or *d*-histidine, or pyruvic acid (143).

When urocanic acid was injected, even in doses as low as 0.1 gm., it was excreted largely unchanged; from urocanic acid solutions incubated with a crude extract of rat liver for 24 hours, *l*-glutamic acid was isolated in theoretical yield (145). As a result of these observations and the decrease in primary amino nitrogen noted in previous tests, such decrease when histidine is added to liver slices being apparently not accompanied by an appreciably accelerated oxygen uptake (147), Edlbacher suggests that certainly the chief route of histidine metabolism is cleavage of the imidazole ring by histidase to produce *l*-glutamic acid without disrupting the α-amino group; but that an alternate and subsidiary mechanism may provide urocanic acid by α-deamination, in which case cleavage by urocanicase may produce *l*-glutamic acid via *l*-isoglutamine, the α carbon of which was originally part of the imidazole ring (143, 145).

Repetition of previous studies has substantiated the earlier observation that reconstituted *d*-amino acid oxidase does not attack *dl*-histidine (148). On the other hand, rat liver apparently does contain an enzyme capable of oxidizing both *l*- and *d*-histidine without rupturing the imidazole ring (149). Unfortunately the latter paper has not been available.

In rats both *l*- and *d*-imidazolelactic acid promoted moderate growth, the former somewhat better than the latter, when the hy-

drolyzate which they supplemented was made deficient in histidine by precipitation with silver oxide, but not when the histidine was precipitated with mercuric sulfate. Supplementation with *l*-histidine promoted growth on either hydrolyzate. The reason for the discrepancy is not yet apparent (150).

Miscellaneous.—The β-alanine found in carnosine has usually been assumed to arise from aspartic acid. Supporting evidence was not obtained by applying a method of β-alanine assay based on the growth of diphtheria bacillus to solutions of aspartic acid or β-aspartylhistidine which had been incubated with homogenized rat liver or kidney, or with muscle strips. Rabbits showed no β-alanine in the blood or urine after the injection of either compound; after β-alanine was injected it could be detected in both (151). A possible mechanism of synthesis of β-alanine from methylglyoxal, suggested by work with yeast, has recently been proposed, apparently with little experimental substantiation (152).

Mice fed glycine which contained C^{13} in the carboxyl position stored more glycogen in the liver than could have been produced from the glycine of the diet. Hence, analytical data which show that the feeding of one compound produces a new and different one do not necessarily justify assuming that the new substance was actually formed from the compound fed; ordinarily the safer interpretation would be the less definite one that the compound fed promoted the formation of the new substance (153).

It is undoubtedly obvious to the reader that any discussion of even the current literature in a field this comprehensive cannot be complete. To keep within the space allotted and yet offer more than a catalogue of references, several interesting aspects of protein metabolism have had to be left untouched. Papers have been omitted which might have contributed to the subjects discussed; perhaps some of those included might better have been omitted. Differences of opinion and judgment are inevitable, but fortunately are often stimulating.

LITERATURE CITED

1. Holt, L. E., Jr., Albanese, A. A., Brumback, J. E., Jr., Kajdi, C., and Wangerin, D. M., *Proc. Soc. Exptl. Biol. Med.*, **48**, 726–28 (1941)
2. Cox, W. M., Jr., Mueller, A. J., and Fickas, D., *Proc. Soc. Exptl. Biol. Med.*, **51**, 303–5 (1942)
3. Dunn, M. S., and Lewis, H. B., *J. Biol. Chem.*, **49**, 327–41 (1921)
4. Toennies, G., *J. Biol. Chem.*, **145**, 667–70 (1942)
5. Albanese, A. A., *Science*, **98**, 46 (1943)
6. Bennett, M. A., and Toennies, G., *J. Biol. Chem.*, **145**, 671–77 (1942)
7. Albanese, A. A., Holt, L. E., Jr., Brumback, J. E., Jr., Hayes, M., Kajdi, C., and Wangerin, D. M., *Proc. Soc. Exptl. Biol. Med.*, **48**, 728–30 (1941)
8. Albanese, A. A., Holt, L. E., Jr., Frankston, J. E., Kajdi, C. N., Brumback, J. E., Jr., and Wangerin, D. M., *Proc. Soc. Exptl. Biol. Med.*, **52**, 209–11 (1943)
9. Albanese, A. A., Holt, L. E., Jr., Brumback, J. E., Jr., Kajdi, C. N., Frankston, J. E., and Wangerin, D. M., *Proc. Soc. Exptl. Biol. Med.*, **52**, 18–20 (1943)
10. Holt, L. E., Jr., Albanese, A. A., Shettles, L. B., Kajdi, C., and Wangerin, D. M., *Federation Proc.*, **1**, 116–17 (1942)
11. Rose, W. C., Haines, W. J., and Johnson, J. E., *J. Biol. Chem.*, **146**, 683–84 (1942)
12. Rose, W. C., Haines, W. J., Johnson, J. E., and Warner, D. T., *J. Biol. Chem.*, **148**, 457–58 (1943)
13. Rose, W. C., *Physiol. Revs.*, **18**, 109–36 (1938)
14. Rose, W. C., and Rice, E. E., *Science*, **90**, 186–87 (1939)
15. Rose, W. C. (Personal communication)
16. Elman, R., Davey, H. W., and Loo, Y., *Arch. Biochem.*, **3**, 45–52 (1943)
17. Bauer, C. D., and Berg, C. P., *J. Nutrition*, **26**, 51–63 (1943)
18. Madden, C. S., and Whipple, W. H., *Physiol. Revs.*, **20**, 194–217 (1940)
19. Beattie, J., and Collard, H. B., *Brit. Med. J.*, **II**, 507–11 (1942)
20. Berryman, G. H., Bollman, J. L., and Mann, F. C., *Am. J. Physiol.*, **139**, 556–62 (1943)
21. Holman, R. L., *J. Exptl. Med.*, **76**, 519–25 (1942)
22. Elman, R., and Davey, H. W., *J. Exptl. Med.*, **77**, 1–5 (1943)
23. Shearburn, E. W., *Surg. Gynecol. Obstet.*, **74**, 343–47 (1942); Holman, R. L., Mahoney, E. B., and Whipple, G. H., *J. Exptl. Med.*, **59**, 269–82 (1934)
24. Elman, R., *J. Am. Med. Assoc.*, **120**, 1176–80 (1942); Elman, R., and Lischer, C., *Surg. Gynecol. Obstet.*, **76**, 503–14 (1943)
25. Elman, R., Charnas, R., and Davey, H. W., *Arch. Surg.*, **47**, 216–20 (1943)
26. Elman, R., and Lischer, C. E., *Ann. Surg.*, **118**, 225–37 (1943)
27. Madden, S. C., Carter, J. R., Kattus, A. A., Jr., Miller, L. L., and Whipple, G. H., *J. Exptl. Med.*, **77**, 277–95 (1943)
28. Robscheit-Robbins, F. S., Miller, L. L., and Whipple, G. H., *J. Exptl. Med.*, **77**, 375–96 (1943)

29. Seligman, A. M., and Fine, J., *J. Clin. Investigation*, **22**, 265–73 (1943)
30. Fine, J., and Seligman, A. M., *J. Clin. Investigation*, **22**, 285–303 (1943)
31. Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exptl. Med.*, **77**, 397–410 (1943)
32. Lischer, C. E., Elman, R., and Davey, H. W., *Am. J. Physiol.*, **139**, 638–41 (1943)
33. Berryman, G. H., and Bollman, J. L., *Am. J. Physiol.*, **139**, 592–95 (1943)
34. Miller, L. L., and Whipple, G. H., *J. Exptl. Med.*, **76**, 421–35 (1942)
35. Gray, S. J., and Barron, E. S. G., *J. Clin. Investigation*, **22**, 191–200 (1943)
36. Brunschwig, A., Scott, V. B., and Moe, R., *Proc. Soc. Exptl. Biol. Med.*, **52**, 46–48 (1943)
37. Little, J. M., and Dameron, J. T., *Am. J. Physiol.*, **139**, 438–45 (1943)
38. Parkins, W. M., and Lockwood, J. S., *Am. J. Med. Sci.*, **205**, 876 (1943); Koop, C E., Riegel, C., Vars, H. M., Ratcliffe, H. L., Parkins, W. M., and Lockwood, J. S., *Am. J. Med. Sci.*, **205**, 876–78 (1943)
39. Goss, H., *Nutrition Abstracts & Revs.*, **12**, 531–38 (1943)
40. Loosli, J. K., and McCay, C. M., *J. Nutrition*, **25**, 197–202 (1943)
41. Owen, E. C., Smith, J. A. B., and Wright, N. C., *Biochem. J.*, **37**, 44–53 (1943)
42. Pearson, R. M., and Smith, J. A. B., *Biochem. J.*, **37**, 148–53 (1943)
43. Pearson, R. M., and Smith, J. A. B., *Biochem. J.*, **37**, 153–64 (1943)
44. Berg, C. P., *Federation Proc.*, **1**, 281–87 (1942)
45. Bovarnick, M., *J. Biol. Chem.*, **145**, 415–24 (1942)
46. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 86–92 (1943)
47. Hotchkiss, R. D., *J. Bact.*, **45**, 64–65 (1943)
48. Christensen, H. N., *J. Biol. Chem.*, **151**, 319–24 (1943)
49. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 313–18 (1943)
50. Abderhalden, E., *Z. physiol. Chem.*, **275**, 135–54 (1942)
51. Abderhalden, E., *Ber. deut. chem. Ges.*, **75**, 1800–2 (1942)
52. Klingmüller, V., *Z. physiol. Chem.*, **278**, 97–119 (1943)
53. Wieland, T., *Ber. deut. chem. Ges.*, **75**, 1001–7 (1942)
54. Kögl, F., Erxleben, H., and van Veersen, G. J., *Z. physiol. Chem.*, **277**, 251–83 (1943)
55. Westphal, U., *Z. physiol. Chem.*, **276**, 191–204 (1942)
56. Westphal, U., and Lang, K., *Z. physiol. Chem.*, **276**, 205–13 (1942)
57. Westphal, U., *Z. physiol. Chem.*, **278**, 213–21 (1943)
58. Westphal, U., *Z. physiol. Chem.*, **278**, 222–29 (1943)
59. Shack, J., *J. Natl. Cancer Inst.*, **3**, 389–96 (1943)
60. Ratner, S., Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, **147**, 549–56 (1943)
61. Albanese, A. A., and Irby, V., *Science*, **98**, 286–88 (1943)
62. Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **148**, 461–62 (1943)
63. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **148**, 249–50 (1943); **151**, 273–80 (1943)
64. Krebs, H. A., *Biochem. J.*, **36**, 758–67 (1942)

65. Leuthardt, F., *Z. physiol. Chem.*, **252**, 238–60 (1938)
66. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **141**, 717–38 (1941)
67. Bach, S. J., *Biochem. J.*, **33**, 1833–43 (1939)
68. Trowell, O. A., *J. Physiol.*, **100**, 432–58 (1941–42)
69. Gornall, A. G., and Hunter, A., *J. Biol. Chem.*, **147**, 593–615 (1943)
70. Gornall, A. G., and Hunter, A., *Biochem. J.*, **35**, 650–58 (1941)
71. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33–66 (1932)
72. Hamilton, P., *J. Biol. Chem.*, **145**, 711 (1942)
73. Harris, M. M., *Science*, **97**, 382–83 (1943); Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Investigation*, **22**, 569–76 (1943)
74. Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Futcher, P. H., and Hiller, A., *J. Biol. Chem.*, **150**, 481–82 (1943)
75. Bliss, S., *Science*, **67**, 515–16 (1928)
76. Bliss, S., *J. Biol. Chem.*, **81**, 129–35, 137–57, 405–19 (1929)
77. Krebs, H. A., *Biochem. J.*, **29**, 1951–69 (1935)
78. McIlwain, H., Fildes, P., Gladstone, G. P., and Knight, C. J. G., *Biochem. J.*, **33**, 223–29 (1939)
79. Handler, P., and Bernheim, M. L. C., *J. Biol. Chem.*, **150**, 335–38 (1943)
80. Simmonds, S., and Vigneaud, V. du, *J. Biol. Chem.*, **146**, 685–86 (1942)
81. Simmonds, S., Cohn, M., Chandler, J. P., and Vigneaud, V. du, *J. Biol. Chem.*, **149**, 519–25 (1943)
82. Schenck, J. R., Simmonds, S., Cohn, M., Stevens, C. M., and Vigneaud, V. du, *J. Biol. Chem.*, **149**, 355–59 (1943)
83. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395–400 (1943)
84. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146**, 357–68 (1942)
85. Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, **150**, 401–6 (1943)
86. Kotake, Y., Ichihara, K., and Nakata, H., *Z. physiol. Chem.*, **243**, 253–56 (1936)
87. Bauer, C. D., and Berg, C. P., *J. Nutrition*, **25**, 497–502 (1943)
88. Jones, D. B., Divine, J. P., and Horn, M. J., *J. Biol. Chem.*, **146**, 571–75 (1942)
89. Stekol, J. A., *Arch. Biochem.*, **2**, 151–57 (1943)
90. Zbarsky, S. H., and Young, L., *J. Biol. Chem.*, **151**, 217–19 (1943)
91. Pearle, D. P., Jr., Smull, K., and Victor, J., *J. Exptl. Med.*, **76**, 317–22 (1942)
92. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **146**, 381–84 (1942)
93. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **149**, 543–48 (1943)
94. Floyd, N. F., and Medes, G., *Arch. Biochem.*, **2**, 135–41 (1943)
95. Lawrence, J. M., and Smythe, C. V., *Arch. Biochem.*, **2**, 225–34 (1943)
96. Binkley, F., *J. Biol. Chem.*, **150**, 261–62 (1943)
97. Fromageot, C., and Desnuelle, P., *Compt. rend.*, **214**, 647–48 (1942)
98. Smythe, C. V., *Arch. Biochem.*, **2**, 259–68 (1943)
99. Blaschko, H., *Biochem. J.*, **36**, 571–74 (1942)
100. Medes, G., and Floyd, N., *Biochem. J.*, **36**, 836–44 (1942)
101. Lanyar, F., *Z. physiol. Chem.*, **275**, 217–24 (1942)
102. Lanyar, F., *Z. physiol. Chem.*, **273**, 283–84 (1942)
103. Lanyar, F., *Z. physiol. Chem.*, **275**, 225–31 (1942)

104. Lanyar, F., *Z. physiol. Chem.*, **278**, 155–64 (1943)
105. Abbott, L. D., Jr., and Salmon, C. L., Jr., *J. Biol. Chem.*, **150**, 339–43 (1943)
106. Closs, K., and Braaten, K., *Z. physiol. Chem.*, **271**, 221–45 (1941), quoted in *Chem. Abstracts*, **37**, 3148 (1943)
107. Abbott, L. D., Jr., *Proc. Soc. Exptl. Biol. Med.*, **49**, 14–15 (1942)
108. Martin, G. J., *Arch. Biochem.*, **1**, 397–401 (1943)
109. Martin, G. J., and Hueper, W. C., *Arch. Biochem.*, **1**, 435–38 (1943)
110. Hueper, W. C., and Martin, G. J., *Arch. Path.*, **35**, 685–94 (1943)
111. Sullivan, M. X., Hess, W. C., and Sebrell, W. H., *U.S. Pub. Health Repts.*, **47**, 75–83 (1932)
112. Lillie, R. D., *U.S. Pub. Health Repts.*, **47**, 83–93 (1932)
113. Sealock, R. R., *J. Biol. Chem.*, **146**, 503–9 (1942)
114. Levine, S. Z., Dann, M., and Marples, E., *J. Clin. Investigation*, **22**, 551–62 (1943)
115. Dann, M., Marples, E., and Levine, S. Z., *J. Clin. Investigation*, **22**, 87–93 (1943)
116. Lang, K., and Westphal, U., *Z. physiol. Chem.*, **276**, 179–90 (1942)
117. Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, **147**, 1–9 (1943)
118. Franklin, A. L., and Chaikoff, I. L., *J. Biol. Chem.*, **148**, 719–20 (1943)
119. Schachner, H., Franklin, A. L., and Chaikoff, I. L., *J. Biol. Chem.*, **151**, 191–99 (1943)
120. Reineke, E. P., Williamson, M. B., and Turner, C. W., *J. Biol. Chem.*, **147**, 115–19 (1943)
121. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, **149**, 555–61 (1943)
122. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, **149**, 563–70 (1943)
123. Abelin, I., *Helv. Chim. Acta*, **25**, 1421–32 (1942), quoted in *Chem. Abstracts*, **37**, 5989 (1943)
124. Morton, M. E., Chaikoff, I. L., Reinhardt, W. O., and Anderson, E., *J. Biol. Chem.*, **147**, 757–69 (1943)
125. Totter, J. R., and Day, P. L., *J. Nutrition*, **24**, 159–66 (1942)
126. Albanese, A. A., and Buschke, W., *Science*, **95**, 584–86 (1942)
127. Cahill, W. M., and Kotalik, G. C., *J. Nutrition*, **26**, 471–76 (1943)
128. Albanese, A. A., Holt, L. E., Jr., Kajdi, C. N., and Frankston, J. E., *J. Biol. Chem.*, **148**, 299–309 (1943)
129. Orten, A. U., and Orten, J. M., *J. Nutrition*, **26**, 21–31 (1943)
130. Albanese, A. A., Randall, R. M., and Holt, L. E., Jr., *Science*, **97**, 312–13 (1943)
131. Alcock, R. S., *Biochem. J.*, **28**, 1721–28 (1934)
132. Kotake, Y., and Iwao, J., *Z. physiol. Chem.*, **195**, 139–47 (1931)
133. Butenandt, A., Weidel, W., and Derjugin, W. v., *Naturwissenschaften*, **30**, 51 (1942)
134. Kotake, Y., *Z. physiol. Chem.*, **270**, 41–96 (1941), quoted in *Chem. Abstracts*, **37**, 146–47 (1943)
135. Lepkovsky, S., and Nielson, E., *J. Biol. Chem.*, **144**, 135–38 (1942)
136. Wintrobe, M. M., Follis, R. H., Jr., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Bull. Johns Hopkins Hosp.*, **72**, 1–25 (1943)

137. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195–201 (1943)
138. Musajo, L., and Minchilli, M., *Ber. deut. chem. Ges.*, **74B**, 1839–43 (1941), quoted in *Chem. Abstracts*, **36**, 5176–77 (1942)
139. Musajo, L., *Gazz. chim. ital.*, **67**, 165–71, 171–78, 179–88 (1937)
140. Musajo, L., and Chiancone, F. M., *Gazz. chim. ital.*, **67**, 218–22 (1937)
141. Musajo, L., and Minchilli, M., *Gazz. chim. ital.*, **70**, 307–10 (1940), quoted in *Chem. Abstracts*, **35**, 3255 (1941)
142. Borchers, R., Berg, C. P., and Whitman, N. E., *J. Biol. Chem.*, **145**, 657–66 (1942)
143. Edlbacher, S., and Bidder, H. v., *Z. physiol. Chem.*, **273**, 163–76 (1942)
144. Edlbacher, S., and Viollier, G., *Z. physiol. Chem.*, **276**, 108–16 (1942)
145. Edlbacher, S., and Heitz, F., *Z. physiol. Chem.*, **276**, 117–25 (1942)
146. Kotake, Y., *Z. physiol. Chem.*, **270**, 38–40 (1941); *Chem. Abstracts*, **37**, 429 (1943)
147. Featherstone, R. M., and Berg, C. P. (Unpublished data)
148. Karrer, P., Koenig, H., and Appenzeller, R., *Helv. Chim. Acta*, **25**, 911–18 (1942)
149. Edlbacher, S., and Grauer, H., *Helv. Chim. Acta*, **26**, 874–82 (1943), quoted in *Chem. Abstracts*, **37**, 5990 (1943)
150. Crookshank, R., and Berg, C. P., *Proc. Iowa Acad. Sci.*, **49**, 289 (1942)
151. Schenck, J. R., *J. Biol. Chem.*, **149**, 111–15 (1943)
152. Enders, C., *Naturwissenschaften*, **31**, 209 (1943)
153. Olsen, N. S., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **148**, 611–18 (1943)

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THE STEROIDS

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This review is limited to the recent chemical work on bile acids, sterols, steroid hormones, and their metabolism. The sapogenins and saponins are not included because of last year's excellent survey.[1] The new developments in the vitamin D field are reviewed elsewhere in this volume (see page 422).

Distribution of Sterols

A systematic investigation of the digitonide precipitate obtained from the nonsaponifiable fraction from marine forms reveals the general distribution of cholesterol, the very frequent occurrence of 7-dehydrosterols, and probably of other known and unknown sterols. The 7-dehydrosterol estimations were based mainly on the absorption spectrum values in the ultraviolet range. The authors conclude that *Meandra areolata* and *Xiphogorgia sp.* contain clionasterol or a substance very similar thereto; *Limulus polyphemus* and three varieties of sea urchin contain clionasterol or a sitosterol type; and *Plexaura flexuosa* contains a mixture of C_{30} and C_{31} steroid compounds, one of which has been named gorgosterol (1).

Cholesterol has been found in the liver of *Sepia officinalis,* but none was found in sea anemone. Relatively pure clionasterol ($C_{27}H_{46}O$) and actiniasterol ($C_{27}H_{44}O$) were separated from *Anemonia sulcata* (2).

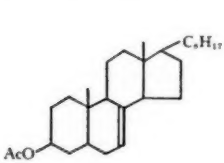
Dehydration of Steroids

Attempts to dehydrate cholesterol by heating a solution thereof in liquid sulfur dioxide with the addition of various catalysts led to the conclusion that seventeen hours at 100° C in the presence of anhydrous copper sulfate forms 54 per cent of dicholesteryl ether and leaves 41 per cent as unchanged; with copper, Raney nickel, ferrous sulfate

[1] *Annual Review of Biochemistry,* XI, 101–50.

and a mixture of sodium carbonate and cupric phosphate no ether was formed; "plaster of Paris inhibits the reaction"; hydrated copper sulfate causes more extensive side reactions; and heating at 135°C causes resinification (3).

Refluxing 3(β)-acetylcholestanol-7(β) in pyridine with *p*-toluene sulphonyl chloride gave the best yield of Δ^7 or γ-cholestenol acetate (I). By oxidizing this with osmium tetroxide and saponifying a cholestanetriol-3(β), 7,8 (II) was obtained. By oxidation with perbenzoic acid and saponification, cholestanediol-3,7-oxide-8,14 (III) was produced, which after conversion into the 3(β)-acetate and oxidation by chromium trioxide gave a 7-keto derivative (IV) and this by hydrochloric acid with ethanol yielded either or both V and VI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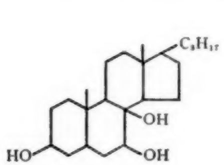
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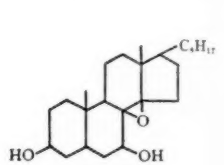
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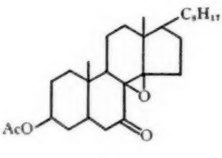
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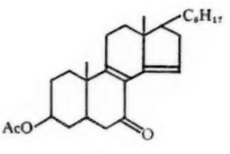
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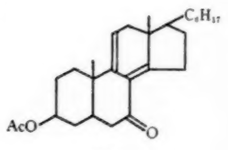
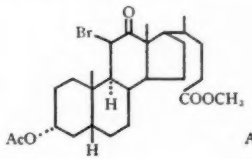

VI

(4). In support of the above structural relations it has been observed that the catalytic reduction of 7-keto-cholesterol acetate produces the two epimeric forms, 7(α) and 7(β) of 3(β)-acetylcholestandiol-7 (5).

In the acetylation of ergosterol by boiling acetic anhydride the yield of acetate is always very low. The main product obtained is the dehydrated derivative, ergostatetraene-B (6).

Interesting observations are accumulating on the dehydration of the 11 or 12-hydroxy derivatives of the bile acids and in the pregnene series. In the bromination of 3(α)-acetyl-12-ketocholanic acid methyl ester two monobromo derivatives are formed. One of these, the 11-

bromo derivative (VII), when debrominated in pyridine yields 3(α)-acetyl-12-ketocholenic-9 acid methyl ester (VIII). Reduction



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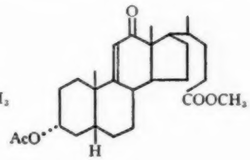
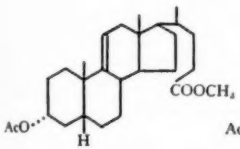
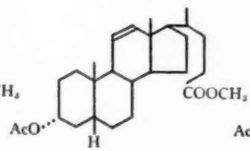


VIII

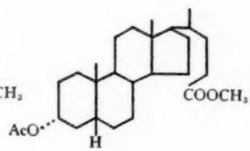
of the latter by the Wolff-Kishner procedure led to the production of three acids which were difficult to separate. On the basis of the types of oxidation products obtained by treatment with perbenzoic acid it is probable that the reduction products obtained were 3(α)-acetyl-cholenic-9:11 acid methyl ester (IX), 3(α)-acetyl-cholenic-11:12 acid methyl ester (X) and lithocholic acid methyl ester (XI) (7).



IX

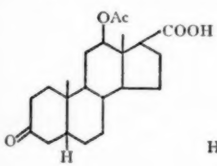


X

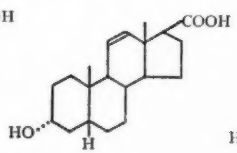


XI

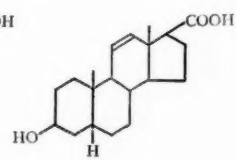
The greater speed of saponification of the 3-acetyl group in 3(α), 12(β)-diacetyl-etiocholanic acid was taken advantage of in the preparation of the 3 keto-12(β)-acetyl-etiocholanic acid (XII). The latter after saponification was benzoylated to yield 3-keto-12-benzoyl-etiocholanic acid and this on heating in high vacuum yielded the 3-keto-etiocholenic-11 acid. Reduction of this yielded 3(α)- and 3(β)-hydroxy-etiocholenic-11:12 acid (XIII and XIV) (8).



XII

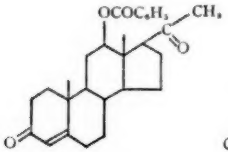


XIII

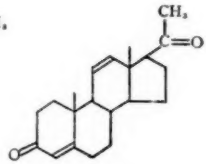


XIV

A dehydration of 12-hydroxyprogesterone through heat treatment of the 12-benzoyl derivative (XV) led to the formation of what probably is an 11 : 12-dehydroprogestrone (XVI) which is quite different from the 9 : 11-dehydroprogesterone prepared previously from 11-hydroxyprogesterone. The new product (XVI) is only about one half as active as progesterone (9).



XV

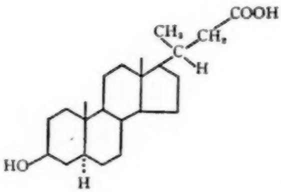


XVI

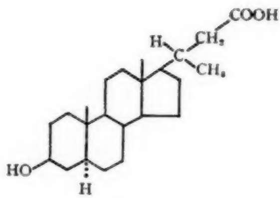
The dehydration of corticosterone acetate by dilute mineral acids has been found to lead to the formation of a mixture of 11(α)- and 11(β)-hydroxy and $\Delta^{9:11}$- and $\Delta^{11:12}$-corticosterone acetate (10).

Hydrogenation of Bile Acids and Sterols

Complete hydrogenation of $\Delta^{5:6,\ 20:22}$-3(β)-hydroxy-norcholadienic acid yielded a mixture of isomers involving carbon 20 (XVII and XVIII) (11).



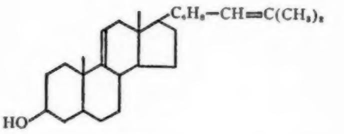
XVII



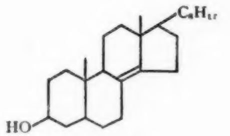
XVIII

The catalytic hydrogenation of dehydrocholic acid with Raney nickel catalyst at 105 to 110° C in the presence of methanol leads to the formation of 12 per cent methyl dehydrocholate, 20 per cent methyl reductodehydrocholate, and 67 to 85 per cent of reductodehydrocholic acid (12).

Hydrogenation of zymosterol (XIX) in the presence of platinum in an acid solution forms mainly α-cholestenol (XX). In other words, the 9:11 double bond is not saturated but shifted to the 8:14 position

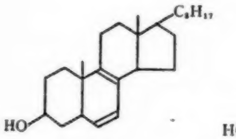


XIX

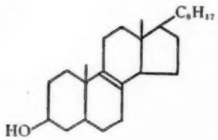


XX

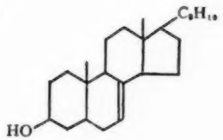
and the side chain becomes completely saturated. In a neutral medium, such as acetic ether, only the unsaturated bond in the side chain becomes hydrogenated but the double bond in the ring remains in the original position. Apparently the 6:7 unsaturated position is easily hydrogenated in acetic acid solutions for isodehydrocholesterol (XXI) is also transformed into α-cholestenol (XX) but in part into δ-cholestenol (XXII). Ergosterol in acid solution in the same way leads to α-ergostenol, but if hydrogenated in neutral media yields $\Delta^{7:8}$ and $\Delta^{22:23}$ dihydroergosterol and finally γ-ergostenol (XXIII). If palladium black is substituted for platinum as the catalyst then, even in neutral solvents, we find a shift of the labile unsaturated bond to the $\Delta^{8:14}$ position (13).



XXI



XXII



XXIII

Bile Acid Preparation

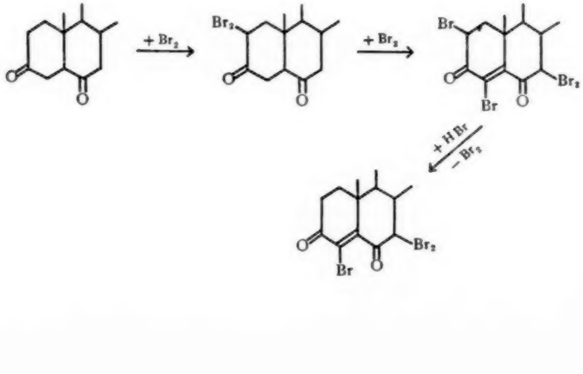
For the preparation of desoxycholic acid the oxidation of cholic acid in 10 *N* acetic acid and sodium acetate by the gradual addition of potassium dichromate at 18 to 20° C during about twenty-four hours has been recommended. The gum after fractionation and reduction yielded 40 to 50 per cent of the weight of gum as desoxycholic acid

(14). Desoxycholic acid has been prepared by the oxidation of cholic acid in acetic acid or a mixture of acetic acid, water, and benzene by addition of chromic acid in dilute acetic acid, the separation of the keto compound formed as a semi-carbazone, and the reduction of the latter by the Wolff-Kishner method (15). Oxidation of cholic acid by chromic acid in glacial acetic acid plus one sixth volume water at 0 to 7° C led to the formation of 3,12-diacetoxy-7-ketocholanic acid. Obviously, the oxidation of the alcohol groups is in the order of the 7, 12, 3 positions (16).

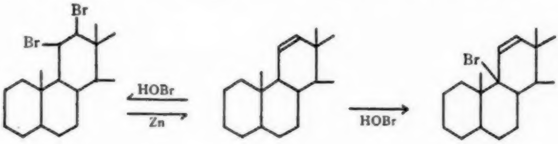
New studies on choleic acids indicate that crystalline choleic acids are formed by combinations of desoxycholic acid with chaulmoogric and hydnocarpic acids, the corresponding dihydro acids and 1,2,5,6-dibenzanthracene, but the ratios of components found do not appear to agree with the prediction of the theory of simple association (17). Bromination of crotonic acid-choleic acid or reduction of acetophenone-choleic acid in various ways did not lead to the formation of optically active compounds. Apparently an asymetric synthesis with choleic acids does not take place (18).

Bromination of Bile Acids and Steroids

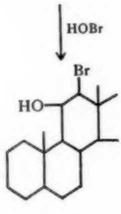
During the past year very important advances have been made in studies involving the bromination of bile acids and steroids followed by the systematic debromination of the bromo derivatives. Thus, in the formation of the tribromide from cholestenedione-3,6 and cholestanedione-3,6 a recent study (19) indicates that the tribromide is formed directly from the former, but that bromination of cholestanedione-3,6 follows the reaction below:



Bromination of various derivatives of Δ^{11}-cholenic acid methyl ester by hypobromite or N-bromoacetamide in tertiary butyl alcohol produces three types of bromo derivatives as indicated below:

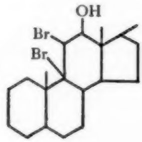


XXV XXIV XXVII



XXVI

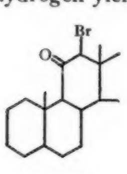
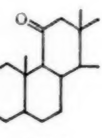
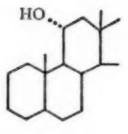
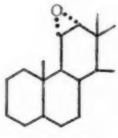
Three general reactions have been shown to hold in the application of these methods of bromination on the methyl esters of Δ^{11}-cholenic acid, 3-keto-Δ^{11}-cholenic acid, 3-acetyl-Δ^{11}-cholenic acid, 3-keto-Δ^{11}-etiocholenic acid and Δ^{11}-pregnenedione-3,20 (20 to 25). The main reaction is the formation of the 11-hydroxy-12-bromo derivative (XXVI). Compounds XXV and XXVII are formed by side reactions. Longer action of hypobromous acid on XXVII forms type XXVIII.



XXVIII

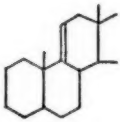
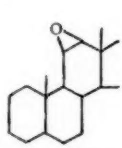
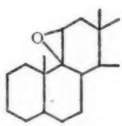
Type XXVI when oxidized by chromium trioxide yields the corresponding 11-keto-12-bromo compound (XXIX). This after debromination by zinc yields XXX which by reduction with platinum plus hydrogen yields the 11-hydroxy derivative (XXXI). If XXVI is treated to remove hydrobromic acid it yields the 11(α),12(α)-oxide

(XXXII), an isomer of XXX, and by reduction with nickel and hydrogen yields in part XXXI. By dehydration of XXXI by hydro-

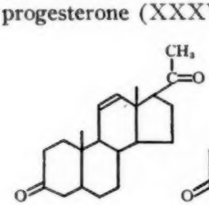
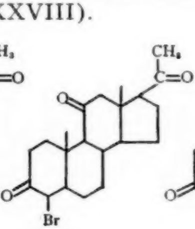
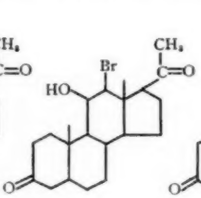
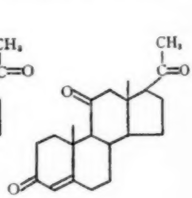
XXIX XXX XXXI XXXII

chloric acid, type XXIV is regenerated with the formation of some of the $\Delta^{9:11}$ derivative (XXXIIa). Oxidation of XXIV by perbenzoic acid yields the 11(β),12(β)-oxide (XXXIII) and the 9,11-oxide (XXXIV) respectively (25).

XXXII*a* XXXIII XXXIV

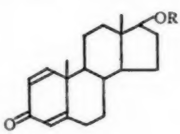
By application of these methods to Δ^{11}-pregnenedione-3,20 (XXXV) the three types of bromo derivatives were formed (24). The 11-hydroxy-12-bromo derivative (XXXVI) when carried through the steps of oxidation, debromination, and rebromination by bromine, under proper conditions, yielded the 4-bromo-11-keto derivative (XXXVII). This when debrominated by pyridine yielded 11-keto-progesterone (XXXVIII).

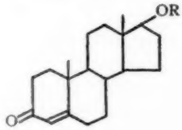
XXXV XXXVII XXXVI XXXVIII

Brominations in the androstane series if done at room temperature and in not too dilute solutions by bromine indicate that the bromine substitutes in different positions depending on the type of ester under consideration (26). Thus, androstanolone-17,3 hexahydrobenzoate or benzoate yields mainly the 2,2-di-bromo derivative, whereas the

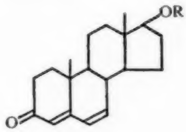
17-acetyl ester of androstanolone-17,3 yields a mixture of the 2,2-dibromo and 2,4-dibromo derivatives. The 2,2-dibromo derivative when treated with collidine yields $\Delta^{1:2}$-2-bromo androstenolone-17,3 esters. These by reduction with zinc yield $\Delta^{1:2}$-androstenolone-17,3. The 2,4-dibromo derivatives similarly treated yield $\Delta^{1:2,4:5}$-androstadienolone esters (XXXIX). These when reduced by zinc yield the $\Delta^{4:5}$-androstenolone-17,3 esters, that is, testosterone esters (XL). The 2,4-dibromo derivatives also form some 2-bromotestosterone esters which on standing in acetic acid plus hydrobromic acid rearranged to 6-bromotestosterone. Treatment of the latter by collidine resulted in the formation of $\Delta^{6:7}$-dehydrotestosterone esters (XLI).



XXXIX



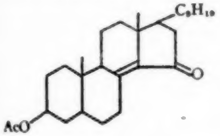
XL



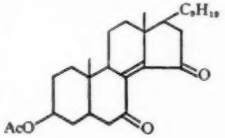
XLI

Oxidation Products without Degradation of the Side Chain

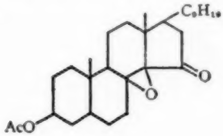
The mild oxidation of α-ergostenyl acetate by chromic acid yielded six neutral oxidation products which on the basis of chemical and spectrographic evidence are interpreted to be keto, oxide, and hydroxy derivatives involving positions 7, 8, 9, 14, and 15 (XLII, XLIII, XLIV, XLV, XLVI, XLVII) (27). Application of the same type



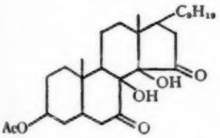
XLII



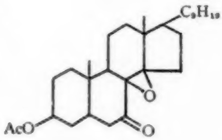
XLIII



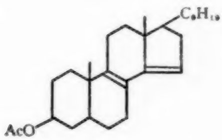
XLIV



XLV



XLVI

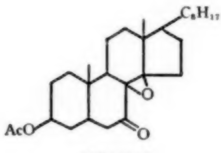


XLVII

of oxidation to α-dihydroergosterol acetate led to the separation of three substances, one of which corresponds to XLII. The other two

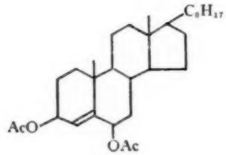
are the 7-keto-8,9-oxide and 7-keto forms. From these studies the authors conclude that α-dihydroergosterol is $\Delta^{8:9,22:23}$-ergostadienol-3 (28). From similar studies carried out on α-spinasterol acetate the authors conclude that α-spinasterol is $\Delta^{8:9,22:23}$-stigmastadienol-3 (29).

Observations on the mild oxidation of α-cholestenyl acetate by chromic acid have also been reported. The main oxidation product is the 7-keto-8:14-oxide derivative (XLVIII) (30).

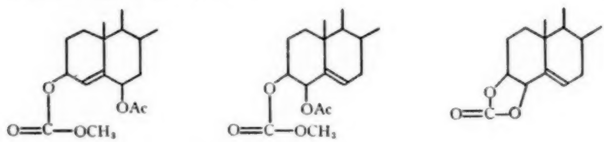


XLVIII

Oxidation of cholesteryl acetate by selenium oxide produced a 25 per cent yield of 3(β),6(β)-diacetyl-Δ^4-cholestene (XLIX). Oxidation of 3-orthocarbomethoxy cholesterol by selenium dioxide in an acetic acid-acetic anhydride medium similarly led to the formation of the following forms (31):



XLIX



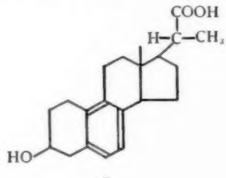
The oxidation of the 3-acid succinates of desoxycholic acid and its homologues yielded the corresponding 12-keto derivatives which on saponification yielded the 3-hydroxy-12-ketocholanic acid and the nor, bisnor, and etio homologues (32).

Of special interest is the oxidation of steroid hormones by N-bromo-acetamide dissolved in tertiary butyl alcohol. A fair yield of androstanedione was obtained from *trans*-androsterone and an 85 per cent yield from androstanediol. Although progesterone was not

affected, desoxycorticosterone probably yielded some Δ^4-pregnenedione-3,20-ol-21 (20).

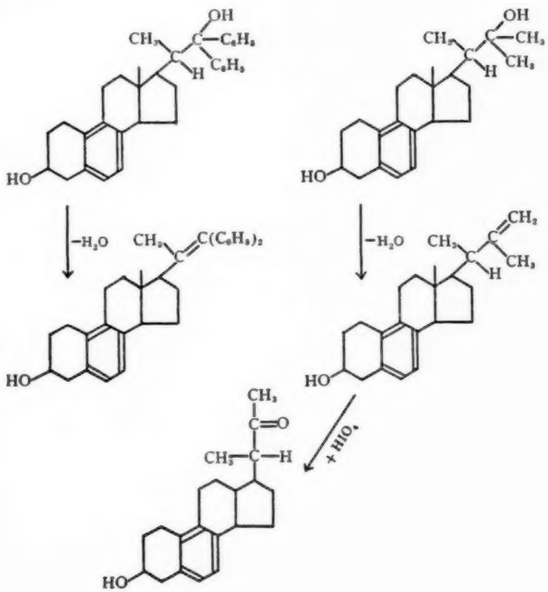
Degradation of the Side Chain

In the degradation of neoergosterol acetate it was found that either ozonolysis or osmic acid and periodic acid probably produce the α-[3(β)-hydroxy-5,7,9-estratrien-17-yl]-propionic acid (L). In ap-

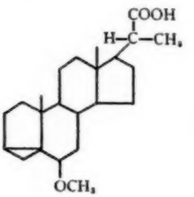


L

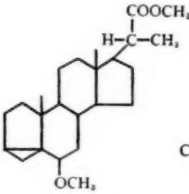
plying the Grignard reaction by adding phenyl magnesium bromide and methyl magnesium iodide respectively to the methyl ester of compound L, two tertiary alcohols were formed which showed a striking difference in their behavior when dehydrated, as indicated below (33).



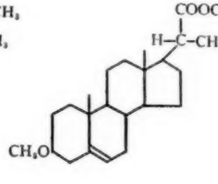
Ozonolysis of *i*-stigmasteryl methyl ether in chloroform at ice bath temperature for two hours yielded 62 per cent of LI which when refluxed with absolute methyl alcohol and potassium acetate produced, in a 98 per cent yield, the methyl ester LII. The methyl ester after refluxing with methanol and sulfuric acid resulted, after rearrangement, in an 87 per cent yield of methyl 3-methoxy-5-bisnorcholenate (LIII) (34).



LI



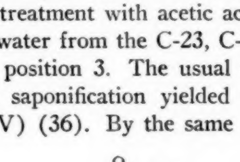
LII



LIII

The oxidation of dibromcholesterol in an alkaline solution by hydrogen peroxide with a trace of moist silver oxide and benzene at 60 to 80° resulted in a yield of 80 mg. of progesterone from 10 gm. of cholesterol (35).

It was expected that application of the Barbier-Wieland degradation to 3-hydroxy-12-ketocholanic acid would leave the 12-keto group intact. With phenyl magnesium bromide a 25 per cent yield of the expected diphenyl tertiary alcohol, with 12-keto group intact, was obtained. This after treatment with acetic acid and acetic anhydride lost one molecule of water from the C-23, C-24 position and was, of course, acetylated in position 3. The usual oxidation by chromium trioxide followed by saponification yielded the 3-hydroxy-12-ketonorcholanic acid (LIV) (36). By the same methods of degradation



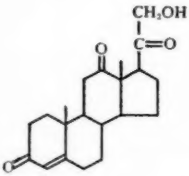
LIV

and with the aid of chromatographic adsorption on aluminum oxide the methyl esters of 3(α)-hydroxy-Δ^{11}-nor- and -bisnorcholenic acids were prepared from 3(α)-hydroxy-Δ^{11}-cholenic acid (37).

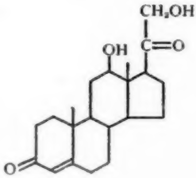
Steroids from or related to the Suprarenal Cortex

References are made elsewhere in this review to studies which have an indirect bearing on this section. Here are presented those studies which have a more specific and direct interest in connection with the chemistry of the suprarenal cortex steroids.

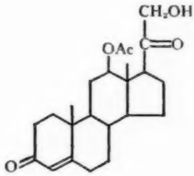
It is well known that the steroids most potent in their effect on carbohydrate metabolism previously separated from the suprarenal cortex contain either the 11-keto or 11-hydroxy group. It was therefore of interest to prepare the corresponding steroids with keto and hydroxy groups in position 12 (LIVa, LV, and LVI). These sub-



LIV*a*



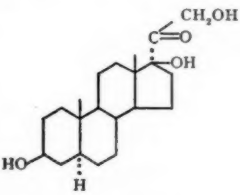
LV



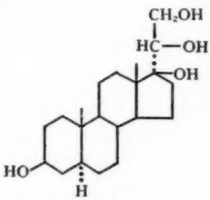
LVI

stances prepared by standard methods from 3(α),12(β)-diacetyletiocholanic acid were found inactive by the Everse–de Fremery test and LVI exhibited no antagonism toward insulin action (38).

Additional evidence on the structures of compounds K and P previously separated from suprarenal extracts has been presented more convincingly and completely. It is now clearly demonstrated that substance P is allopregnanetriol-3(β),17(β),21-one-20 (LVII). Reduction by hydrogen with platinum yields substance K or allo-pregnanetetrol-3(β),17(β),20(β),21 (LVIII). Other important evidence is obvious from the reactions indicated on page 276 (39).

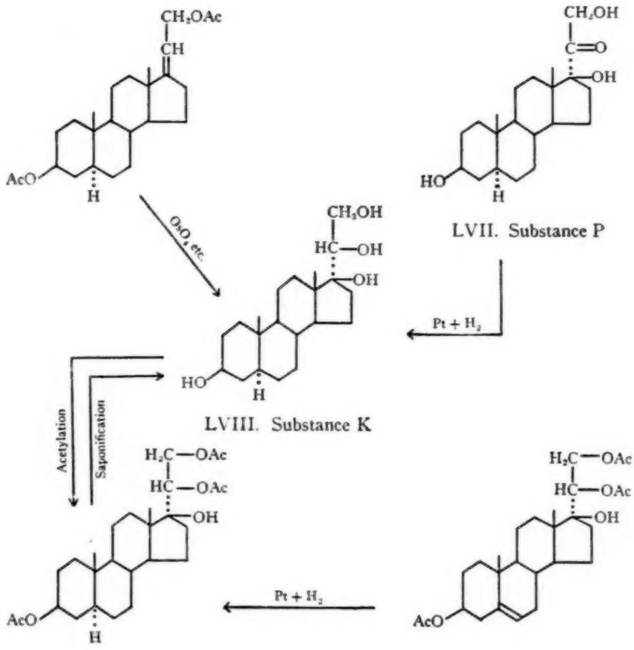


LVII



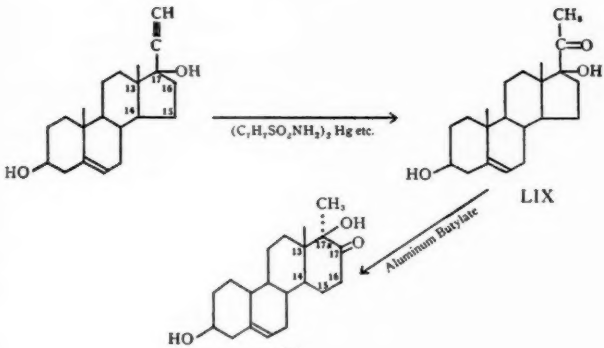
LVIII

In the attempts to prepare 17-hydroxy compounds from the 17-ethinyl series it has been found difficult to control the hydration necessary for this reaction. It is now reported that *p*-toluolsulfamide mercury [$(C_7H_7SO_2NH_2)_2Hg$] is an excellent reagent for this purpose. The resulting 17-ol-20-one (LIX) when treated with aluminum butylate yielded the D-homo form (LX; see page 277). This is true starting with Δ^5-17-ethinylandrostene-3(β),17(α)-diol, 17-ethinyltestosterone, or 17-ethinylandrostane-3(β),17(α)-diol (40).



LVII. Substance P

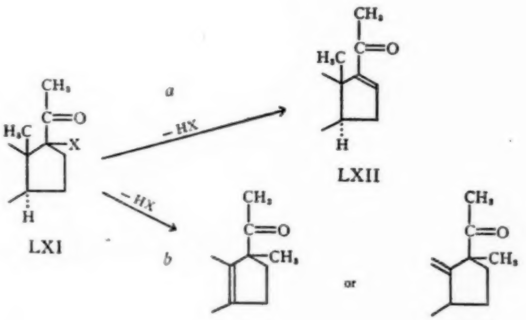
LVIII. Substance K



LIX

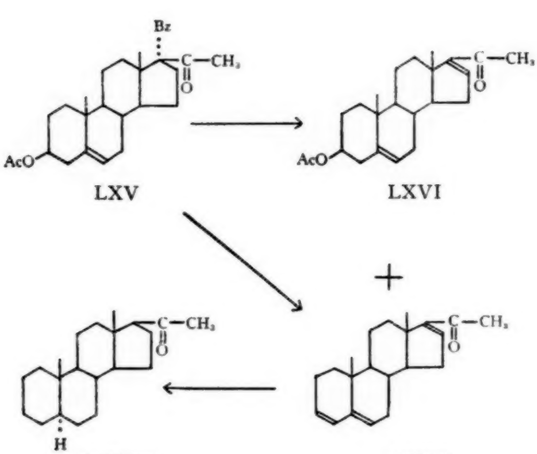
LX

Studies on possible rearrangements in the removal of HX in steroids of the X-17 type where X may represent hydroxyl, acetyl, benzoyl, or chlorine suggest reactions as represented below by paths *a* and *b*.

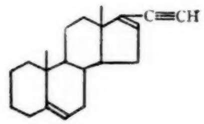


LXI LXII LXIII LXIV

With 3,17-diacetate of pregnenolone-20 these paths were not demonstrated, but melting and vacuum distillation of 3-acetate-17-benzoate of pregnenolone-20 (LXV) reaction *a* was accomplished with the formation of by-products LXVII and LXVIII while no evidence was found for reaction *b*:

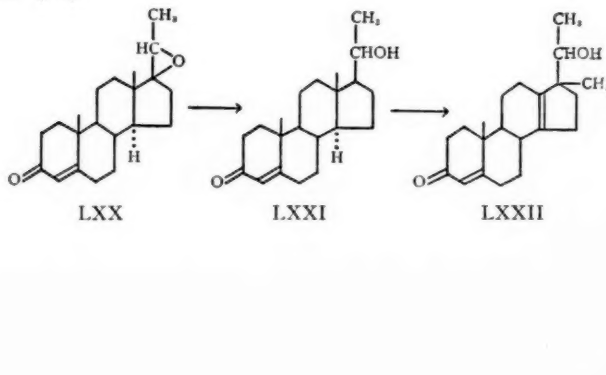


LXV LXVI LXVIII LXVII



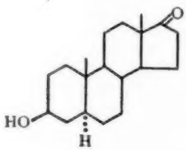
LXIX

Treatment of 3-acetylpregnenol-17-one-20 by melting and distilling does not remove the water, but heating in a sealed tube with phosphorus oxychloride at 100° indicated that route *a* was followed. The same is true for 17-ethinyl-3-acetylpregnenol-17 where in addition to substance LXVI product LXIX was formed. However when 17,20-oxido-Δ^4-pregnenone-3 (LXX) is treated with glacial acetic acid at room temperature reaction *b* seems to take place as indicated below (41).



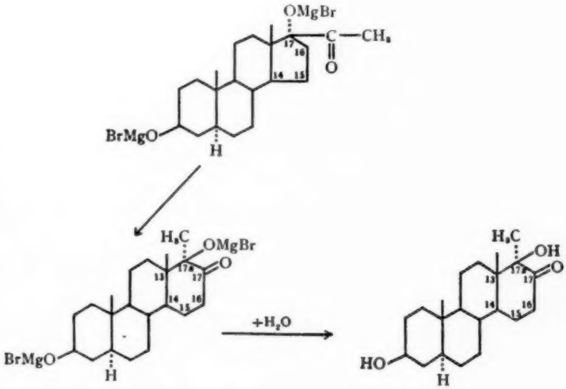
LXX LXXI LXXII

In the treatment of the diacetyl-3,17-allopregnanolone-20 with methyl magnesium bromide and with subsequent hydrolysis the reaction progressed along two paths. The first is the normal one which after acetylation, oxidation, and saponification led to a good yield of *trans*-androsterone (LXXIII). The second involved rearrangements

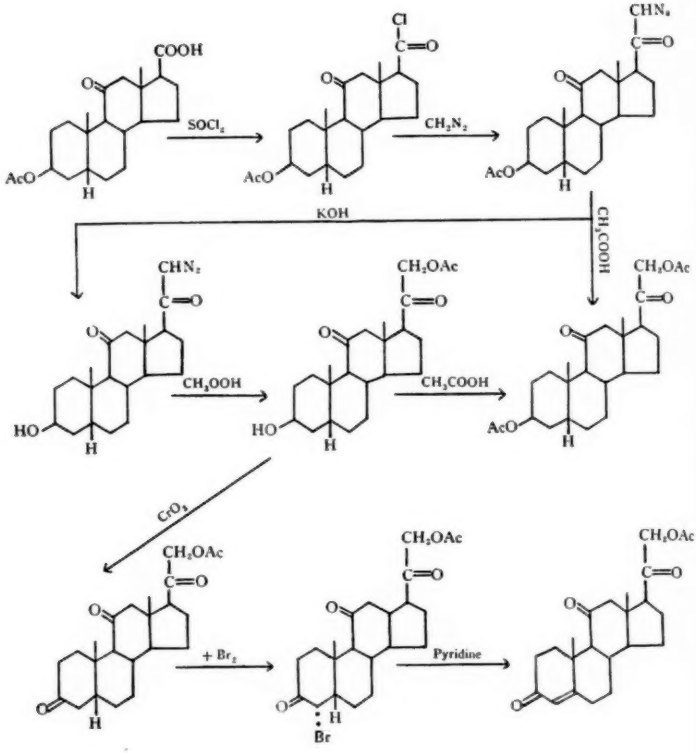


LXXIII

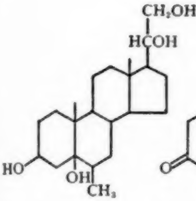
resulting in the formation of D-homo derivatives indicated as follows (42):



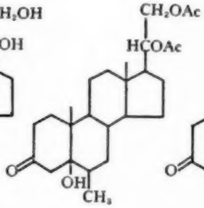
New compounds related to desoxycorticosterone have been prepared and the partial synthesis of 11-dehydrocorticosterone has been accomplished. Perbenzoic acid oxidation of Δ^5-pregnenetriol-3(β),20,21 yielded pure pregnaneoxide-5,6-triol-3(β),20,21. With methyl magnesium bromide this oxide gave 6-methylpregnanetetrol-3(β),5,20,21 (LXXIV). The possible formation of 6-methylpregnanetetrol-3(β),6,20,21 is obvious, but no evidence is given that


COOH
SOCl2
Cl
C=O
CH2N2
CHN2
AcO
H
KOH
CH3COOH
CH3OOH
CH2OAc
HO
CrO3
+ Br2
Br
Pyridine

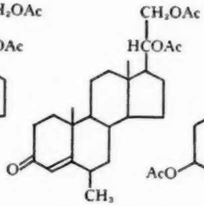
such a derivative was formed. The product LXXIV on acetylation yielded the 21-mono- and the 3,21-diacetate derivatives. From the former the authors obtained by chromic acid oxidation in acetic acid the 20,21-diacetate of 6 methylpregnanetriol-5,20,21-one 3 (LXXV). This when dehydrated by hydrochloric acid gave the corresponding pregnene form (LXXVI). Oxidation of the 3,21-diacetate of compound LXXIV probably led to the formation of the 20-keto derivative (LXXVII) (43).



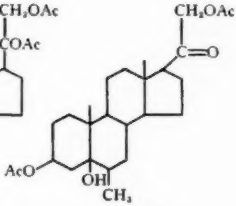
LXXIV



LXXV



LXXVI

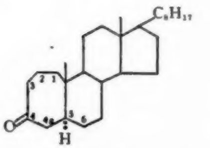


LXXVII

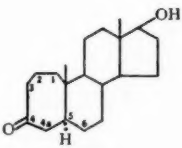
With 3(β)acetyl-11-ketoetiocholanic acid as the starting material the synthesis of 11-dehydrocorticosterone was accomplished by the steps shown on the opposite page (44).

A AND D HOMOSTEROIDS

A-homocholestanone (LXXVIII) and A-homodihydrotestosterone (LXXIX) have been produced (45). The latter is only one twentieth as androgenic as the D-homodihydrotestosterone in the capon test.



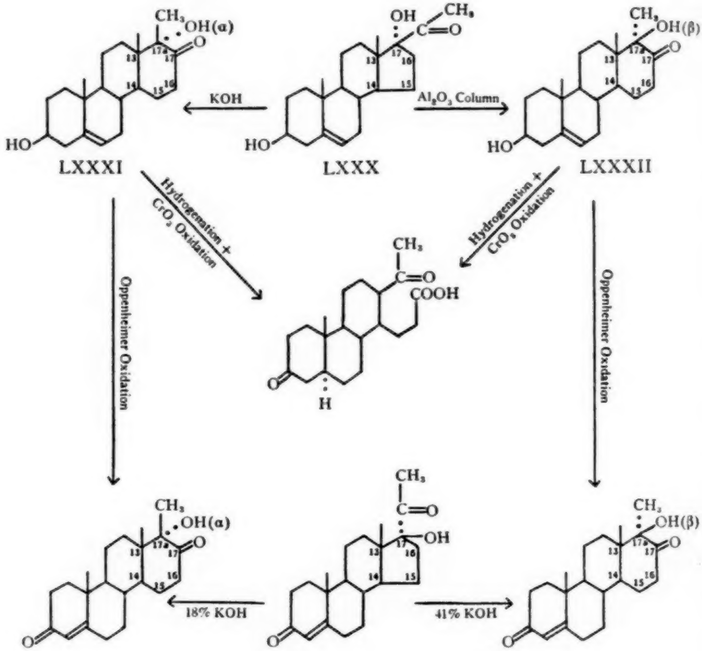
LXXVIII



LXXIX

When 3,17-hydroxy-20-keto-pregnene (LXXX) is treated by potassium hydroxide and aluminum oxide respectively, two different D-homosteroids (polyhydrochrysenes) are formed (LXXXI and LXXXII) in which the α and β configuration on the 17a carbon atom is involved. The preponderance (70 per cent) of the formation of the

β type over the α type (40 per cent) as diacetates is determined by the strength of potassium hydroxide. Low alkalinity favors the α formation, but strong potassium hydroxide or aluminum oxide favors β formation. These relations are indicated below (46, 47).



Two kinds of D-homoandrostane derivatives (LXXXV and LXXXVI) can be obtained by appropriate means from 3(β)-acetyl-17-hydroxy-17-aminomethylandrostane (LXXXIV). These when saponified and oxidized by chromic acid yield two different diketones (LXXXVII and LXXXVIII). However, complete reduction of the two ketone groups in the two isomers to CH_2 groups leads to the production of the same homoandrostane (LXXXIX). This shows that during the rather involved process of converting the 5-carbon D ring into the 6-carbon D ring there are no changes in the relations of rings D and C to each other. The various steps are shown in Figure 1 (48).

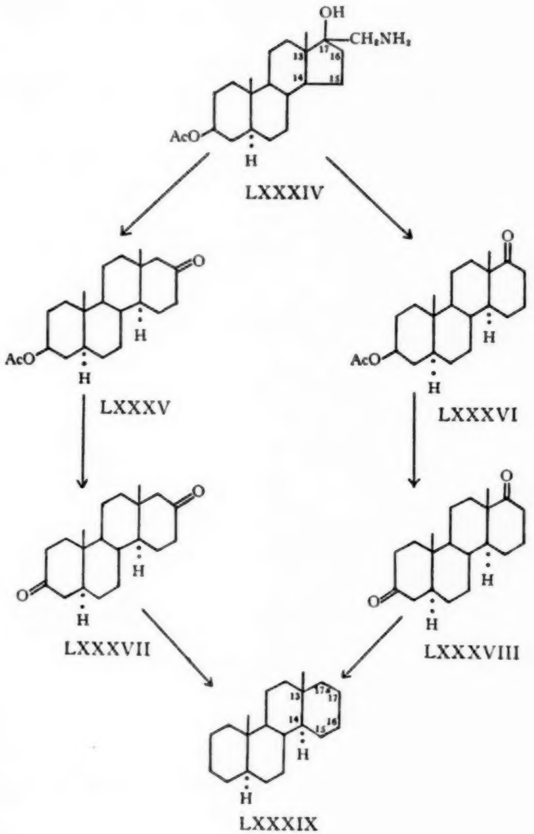

OH
CH_2NH_2
AcO
H
LXXXIV
AcO
H
H
LXXXV
AcO
H
H
LXXXVI
H
H
LXXXVII
H
H
LXXXVIII
H
H
LXXXIX

FIGURE 1

Comparative assays with capons on the androstene or androstane series with the D-homo series are given in Table I. It is of particular interest to note that in shifting the 17-keto group to position 17a in D-homoandrostanedione there is a marked change in physiological activity. The D-homoandrostanediols-3,17 are more potent than the regular forms (48).

TABLE I

RELATIVE ANDROGENIC ACTIVITIES OF THE REGULAR ANDROSTANE AND ANDROSTENE SERIES AND THEIR D-HOMOLOGUES

Substance	Micrograms equivalent to an international unit	
	Regular series μg.	D-homo-series μg.
Testosterone	15	...
17(α) and 17a(α) acetates	20	Very active
Dihydrotestosterone		
17(α)-*trans* and 17a(α)	20	25
17-*cis*	300	...
Androsterone		
cis-3(α)-17a-keto	100	90 to 100
trans-3(β)-17a-keto	300	About 150
Androstanedione-3,17	130	...
D-Homoandrostanedione-3,17a	...	160
D-Homoandrostanedione-3,17	...	300
Androstanediol-3,17		
3(β),17(α)	500	...
D Homo-3(β),17a(α)	...	160
3(β),17(β)	800 to 1000	...
D Homo-3(β),17a(β)	...	500

SYNTHETIC STUDIES

Further advances have been made in the synthetic development of steroid-like substances from simple organic compounds. Thus, a number of 3-keto-1,2-cyclopentenophenanthrenes with different degrees of hydrogenation in rings C and D have been prepared relatively easily. A 6-carbon D ring compound has also been added to the series. None of these substances contain the angular methyl groups (49).

Acylphenanthrene after reduction and conversion into acid bromides was condensed with sodium malonic ester. The acid chloride of the condensation product by treatment with aluminum chloride formed

the 4-ketopentene ring and this by the Clemmensen reduction gave a homologue of Diels' hydrocarbon (50). See Figure 2.

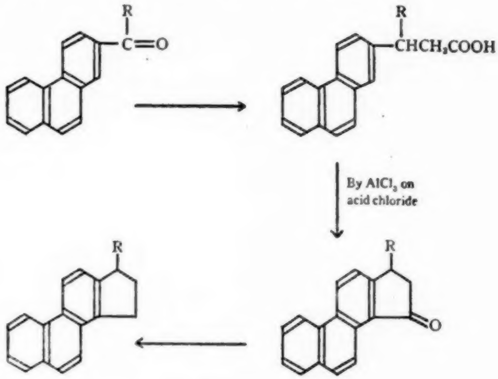


FIGURE 2

A new approach has been attempted starting with the diketone as in Figure 3 and interacting with 4-diethylaminobutane-2-one-methiodide to produce an isomer of androstenedione. A fractional high vacuum distillation gave a product which resembles androstenedione in many respects, especially in absorption spectrum. No positive biological tests have been reported (51).

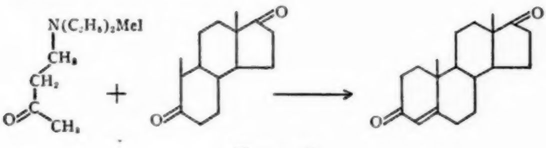


FIGURE 3

COLOR REACTIONS

Modifications of old color reactions have been made mainly with the view of determining their specificities and their possible value for quantitative application. The value of various modifications of the Zimmermann *meta*-dinitrobenzene reaction continues to be a question especially as applied to urine extracts. The 17-ketosteroid estimation by this method is no doubt made much more specific and quantitative by applying it to the neutral ketone fraction (52 to 55).

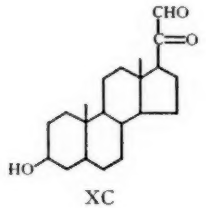
A modification of the antimony trichloride reaction in glacial acetic acid and acetic anhydride has been much more specific for 17-ketosteroids than the Zimmermann reaction. However, dehydroisoandrosterone reacts very faintly and androstanedione gives a negative test (56).

Careful control of a modification of the Pettenkofer reaction with furfural is of value in distinguishing dehydroisoandrosterone from androsterone, testosterone, and methyl testosterone (57).

The application of a modification of the guaiacol sulfonic acid plus sulfuric acid reaction has been found of value in distinguishing estrone and estradiol from estriol. The latter gives a very faint reaction. The method has not been developed for application to urine (58). The importance of details in the control of time, temperature, and concentrations of reagents and water has again been emphasized in the estimation of cholesterol by the Liebermann-Burchard reaction (59).

Steroids in Hog-Testis Tissue

An intensive study on various fractions obtained from the acetone and benzene soluble fractions from hog testes revealed the presence of the following steroids in addition to cholesterol: Δ^5-pregnenol-3(β)-one-20, allopregnanol-3β-one-20, cholestanetriol-3β,5,6(*trans*),-$\Delta^{3,5}$-cholestadieneone-7, and two unidentified substances with the formula $C_{21}H_{32}O_3$ and $C_{13}H_{22}O_2N_2$. The $C_{21}H_{32}O_3$ yielded a dioxime. Neither testalolon (XC) nor testosterone were isolated, but it is possible that these were destroyed during the alkaline hydrolysis. The product reported here is not identical with Hirano's testalolon (60) although it has the same formula, $C_{21}H_{32}O_3$, and like testalolon forms a dioxime. Two isomeric forms of the structure previously ascribed to testalolon were prepared. These also differed from Hirano's product (61).



XC

Metabolism of Sterols

In attempts to prove the hypothesis that cholestenone is an intermediate product in the formation of coprosterol from cholesterol, dogs and rats were placed on a high cholesterol diet by feeding "steamed sheep's brain." By this procedure the feces contained more cholestenone than on the regular diet (62) and hence the authors conclude that these results are in harmony with the hypothesis.

Peat soils are richest in sterols, but sandy soil, grass-land soil, and light loam are very low in sterol content. When cholesterol is added to soil it gradually disappears in twelve months. This is not the case when mercuric chloride is added to the soil. Hence, it is concluded that there is a microbiological degradation of steroids (63).

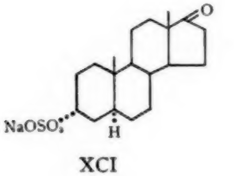
Evidence for the conversion of cholesterol into cholic acid in the dog has been obtained by injecting deuteriocholesterol and following the deuterium distribution in the cholic acid obtained from the gall bladder as well as in the cholesterol extracted from the tissues. The fact that the cholic acid collected from the gall bladder was found to reveal a deuterium content of the same order as the deuteriocholesterol administered indicates that cholesterol was converted into bile acid. The highest concentration of deuteriocholesterol was found in the lung, with liver next, but none in the brain or spinal cord (64). The deuteriocholesterol was prepared *in vitro* by heating cholesterol at 123 to 127° C for hours or days with reduced platinum oxide or selenium in water plus heavy water and/or deuterioacetic acid (65).

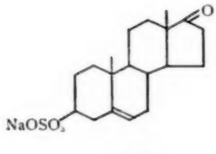
Metabolism of Steroid Hormones

It has been confirmed that part of the suprarenal cortical hormones or products derived therefrom are excreted in human urine. In the "alkali insoluble fraction" obtained from a benzene or chloroform extract is found a principle which inhibits the water intoxication symptoms observed in adrenalectomized rats (66). This fraction not only prolongs the life of adrenalectomized rats but also raises the glycogen content in their livers (67).

Considerable advances have been made in the studies on the separation of conjugated steroids from urine and the application of these findings in improving the methods of extraction of and hydrolysis to the free steroid form. After the separation of androsterone sulfate (XCI) as a sodium salt from a specimen of pathological urine (68) which was exceedingly rich in ketosteroids, due to a liver tumor, it

was of considerable interest to find normal human urine to contain practically all of its neutral 17-ketosteroid fraction in conjugated forms and much of it as the sodium salt of dehydroisoandrosterone sulfate (XCII) (69). The fact that such substances when subjected to acid


XCI


XCII

hydrolysis led to the production of artifacts as by-products was expected in the light of earlier quantitative studies on the extraction of steroids from urine.

Attempts to hydrolyze these sulfates by treatment with barium chloride have been quite successful. Sodium dehydroisoandrosterone sulfate has been hydrolyzed by boiling it in aqueous solution with an excess of barium chloride. This procedure has also been applied to urine extracts (70). The enzymatic hydrolysis of pregnanediol glucuronidate by rat liver acetone powder has been demonstrated and suggests the application of hydrolytic enzymes to the problem of hydrolyzing the other conjugated steroid forms (71). It is, however, obvious that bacterial contamination must be guarded against in order to avoid the formation of still other artifacts.

The estimation of the excretion rate of sodium pregnanediol glucuronidate and its various sources and precursors continues to grow in interest. As a result of studying the distribution coefficient of sodium pregnanediol glucuronidate between neutral or alkaline urine and normal butanol it is now recommended that the quantitative extraction be based upon such distribution coefficients rather than on the tedious complete extraction (72). Studies on rabbits show that the normal animal does not excrete detectable amounts of pregnanediol glucuronidate but after the injection of 225 to 930 mgs. of desoxycorticosterone a 5.6 to 14.6 per cent conversion into pregnanediol glucuronidate takes place (73). These results are not surprising, but it is obvious that more sensitive methods for estimating pregnanediol are needed to study such metabolic changes under normal physiological conditions and to extend these studies to other cortical steroids.

A new steroid glucuronidate has been separated from the urine of a young woman with masculinism. It is not precipitated by digitonine and does not give a positive test for the 17-hydroxyl group. It does not give a typical 17-keto test and probably contains an α-hydroxyl in position 3 and a ketone group in position 20 (74).

Studies on the metabolism of estrogens have been continued by following the total estrogenic activity in the urine and the distribution thereof between fractions representing estrone, estradiol, and estriol. The total recoveries in the urine are very poor and the methods for determining the individual types of estrogen are only approximate. Nevertheless the data indicate that in men and women the reaction estradiol $\rightleftharpoons$ estrone $\rightarrow$ estriol holds. When large amounts of estrone are injected the main end product is estriol with some α-estradiol. Estriol injection is followed by a recovery of 56 per cent of the activity in the estriol fraction, very small amounts as α-estradiol and a doubtful recovery of estrone activity. Injection of α-estradiol in a man resulted in the formation of estrone and estriol but with very poor total recovery (75, 76, 77).

That the liver is the main organ in which this metabolism of estrone and estradiol takes place is confirmed by *in vitro* studies with tissues from rabbits and rats. Activation of estrone on the other hand is achieved by incubation with the endometrium, spleen, heart, and lung. This probably involved the formation of α-estradiol. These general conclusions have been found to hold for normal as well as pregnant animals (78). In perfusion studies of rat livers with estrogens, most activity was recovered as estradiol activity, then almost the same recovery for the estrone type, and only about one half as much in the estriol fraction (79). No evidence was obtained for the conversion of estrone into estradiol from studies on surviving bovine or human endometrium. With rabbit endometrium there were marked losses of estrone, but no evidence of α-estradiol formation (80).

Quite disturbing evidence has been obtained in dogs after the intravenous injection of α-estradiol. It is stated that little or no estrogenic activity is found in the liver three to forty-eight hours after the injection of 250,000 international units of α-estradiol, but that twenty-four to forty-eight hours after injection a considerable amount is found in the gall bladder bile and none in the spleen, intestinal wall, or hepatic vein blood. The authors offer the hypothesis that estrogens are taken up in an inactivated form by liver cells, then re-ex-

creted and found partly active in the bile, and finally returned to the liver after absorption through the portal system (81).

The fact that spayed mice in which fatty livers have been induced by feeding inadequate diet are not more sensitive to the injection of estrogens is taken as evidence that the rate of metabolism of estrogens is not altered in these animals. Naturally one wonders whether the doses of estrogen required for such studies are not too small for the detection of differences in rates of destruction (82). However, it has again been shown that estrone pellets when implanted in the spleens of male rabbits do not produce degeneration of the testicles, such as is observed after subcutaneous implantation (83). The unexpected similarity of stilbestrol to natural estrogen in biological reactions is also found in the destruction of stilbestrol by rat liver pulp (84). Although it has been claimed that vitamin B deficiency in rats results in a lowered inactivating power or function of their livers, this is reported not to be the case as to the destruction or inactivation of testosterone propionate (85).

Studies on the qualitative and quantitative distribution of various steroids in the neutral fraction obtained from the extraction of hydrolyzed urine continue to produce interesting and significant results in health and disease. In three cases of cancer of the breast, no striking increases were found in the excretion of the steroids in the neutral fraction. In fact the amounts of androsterone and 3(α)-hydroxyetiocholanone-17 isolated were abnormally low as compared with the results obtained by others on normal urines (86). On the other hand in the case of an adrenalcortical carcinoma in a seven-year-old boy a marked increase in the excretion of 17-ketosteroids was observed and a new substance, probably Δ^5-androstenetriol-3(β),16,17 was isolated (87). Marked fluctuations in the urinary excretion of gonadotropins, estrogens, and 17-ketosteroids over a period of three months have been observed in four normal men. No correlation between the three constituents was observed (88). A diurnal rhythm in the urinary excretion of 17-ketosteroids was, however, observed in young men—during the day the excretion of 17-ketosteroids per hour was distinctly higher. Usually the volume of urine was also greater during the day, but not regularly. Furthermore an induced diuresis did not increase the rate of 17-ketosteroid excretion (89). Some striking observations have been made on the urinary excretion of 17-ketosteroids in pilots under varying conditions of stress, altitude, and oxygen tension. In all cases "flight conditions" caused 17-ketosteroiduria and diuresis and these

were directly proportional to the percentage of "flying time" for pilots, and greater for test pilots. Poorer "performers" tended to give greater increases in 17-ketosteroiduria and diuresis. It appears that the 17-ketosteroiduria may in part cause some diuresis (90). By following the androgen excretion in normal individuals with the chick comb weight method, 8.4 to 16 international units of androgen per day were found as compared with the older values of 30 to 100 international units. The authors conclude that androgen excretion is constant per liter of urine in a given individual (91). The relation of age and development to the urinary excretion of 17-ketosteroids has been investigated. In children six years to ten years of age there is a steady but slow rise in the excretion. After that the rise is more rapid, especially in boys. Precocious puberty and retarded puberty are usually associated with rises and falls respectively in 17-ketosteroid excretion. More rapid growth usually is associated with rises in the excretion of 17-ketosteroids (92).

LITERATURE CITED

1. Bergmann, W., McLean, M. J., and Lester, D., *J. Org. Chem.*, **8**, 271–84 (1943)
2. Deffner, M., *Z. physiol. Chem.*, **278**, 165–68 (1943)
3. Levin, R. H., *J. Am. Chem. Soc.*, **65**, 627–28 (1943)
4. Wintersteiner, O., and Moore, M., *J. Am. Chem. Soc.*, **65**, 1507–13 (1943)
5. Wintersteiner, O., and Moore, M., *J. Am. Chem. Soc.*, **65**, 1503–7 (1943)
6. Stansbury, H. A., Jr., *J. Am. Chem. Soc.*, **65**, 1243 (1943)
7. Seebeck, E., and Reichstein, T., *Helv. Chim. Acta*, **26**, 536–62 (1943)
8. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 607–19 (1943)
9. Hegner, P., and Reichstein, T., *Helv. Chim. Acta*, **26**, 715–21 (1943)
10. Shoppee, C. W., and Reichstein, T., *Helv. Chim. Acta*, **26**, 1316–28 (1943)
11. Plattner, A., and Pataki, J., *Helv. Chim. Acta*, **26**, 1241–52 (1943)
12. Hoehn, W. M., and Ungnade, H. E., *J. Am. Chem. Soc.*, **65**, 124 (1943)
13. Wieland, H., and Benend, W., *Ann.*, **554**, 1–8 (1943)
14. Haslewood, G. A. D., *Biochem. J.*, **37**, 109–12 (1943)
15. Schneider, A. W., and Hoehn, W. M., *J. Am. Chem. Soc.*, **65**, 485 (1943)
16. Gallagher, T. F., and Long, W. P., *J. Biol. Chem.*, **147**, 131–34 (1943)
17. Bun-Hoi, N. P., *Z. physiol. Chem.*, **278**, 230–35 (1943)
18. Reid, C. C., and Sturtevant, J. M., *J. Am. Chem. Soc.*, **65**, 125 (1943)
19. Sarett, L. H., Chakravorty, P. N., and Wallis, E. S., *J. Org. Chem.*, **8**, 405–16 (1943)
20. Reich, H., and Reichstein, T., *Helv. Chim. Acta*, **26**, 562–85 (1943)
21. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 586–98 (1943)
22. Press, J., Grandjean, P., and Reichstein, T., *Helv. Chim. Acta*, **26**, 598–606 (1943)
23. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 705–15 (1943)
24. Hegner, P., and Reichstein, T., *Helv. Chim. Acta*, **26**, 721–29 (1943)
25. Ott, G. H., and Reichstein, T., *Helv. Chim. Acta*, **26**, 1799–815 (1943)
26. Inhoffen, H. H., and Zühlsdorff, G., *Ber. deut. chem. Ges.*, **76**, 233–46 (1943)
27. Stavely, H. E., and Bollenbeck, G. N., *J. Am. Chem. Soc.*, **65**, 1285–89 (1943)
28. Stavely, H. E., and Bollenbeck, G. N., *J. Am. Chem. Soc.*, **65**, 1290–94 (1943)
29. Stavely, H. E., and Bollenbeck, G. N., *J. Am. Chem. Soc.*, **65**, 1600–3 (1943)
30. Wintersteiner, O., and Moore, M., *J. Am. Chem. Soc.*, **65**, 1513–16 (1943)
31. Paige, M. F. C., *J. Chem. Soc.*, 437–41 (Sept., 1943)
32. Schwenk, E., Riegel, B., Moffett, R. B., and Stahl, E., *J. Am. Chem. Soc.*, **65**, 549–51 (1943)
33. Jacobsen, R. J., *J. Am. Chem. Soc.*, **65**, 1789–92 (1943)
34. Riegel, B., Meyer, E. W., and Beiswanger, J., *J. Am. Chem. Soc.*, **65**, 325–28 (1943)

35. Serono, C., and Marchetti, E., *Gazz. chim. ital.*, **72**, 151 (1943); *Chem. Abstracts*, **37**, 2742 (1943)
36. Riegel, B., and Moffett, R. B., *J. Am. Chem. Soc.*, **65**, 1971–73 (1943)
37. Grandjean, P., and Reichstein, T., *Helv. Chim. Acta*, **26**, 482–92 (1943)
38. Fuchs, H. G., and Reichstein, T., *Helv. Chim. Acta*, **26**, 511–30 (1943)
39. Koechlin, B., and Reichstein, T., *Helv. Chim. Acta*, **26**, 1328–34 (1943)
40. Goldberg, M. W., Aeschbacher, R., and Hardegger, E., *Helv. Chim. Acta*, **26**, 680–86 (1943)
41. Shoppee, C. W., and Prins, D. A., *Helv. Chim. Acta*, **26**, 1004–16 (1943)
42. Shoppee, C. W., and Prins, D. A., *Helv. Chim. Acta*, **26**, 2089–95 (1943)
43. Ehrenstein, M., *J. Org. Chem.*, **8**, 83–94 (1943)
44. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 747–55 (1943)
45. Goldberg, M. W., and Kirchensteiner, H., *Helv. Chim. Acta*, **26**, 288–301 (1943)
46. Shoppee, C. W., and Prins, D. A., *Helv. Chim. Acta*, **26**, 185–200 (1943)
47. Shoppee, C. W., and Prins, D. A., *Helv. Chim. Acta*, **26**, 201–23 (1943)
48. Goldberg, M. W., and Wydler, E., *Helv. Chim. Acta*, **26**, 1142–55 (1943)
49. Bachmann, W. E., Gregg, R. A., and Post, E. F., *J. Am. Chem. Soc.*, **65**, 2314–18 (1943)
50. Riegel, B., Gold, M. H., and Kubico, M. A., *J. Am. Chem. Soc.*, **65**, 1772–76 (1943)
51. Martin, R. H., and Robinson, R., *J. Chem. Soc.*, 491–97 (Oct., 1943)
52. Nathanson, I. T., and Wilson, H., *Endocrinology*, **33**, 189–203 (1943)
53. Engstrom, W. M., and Mason, H. L., *Endocrinology*, **33**, 229–36 (1943)
54. Hansen, L. P., Cantarow, A., and Paschkis, K. E., *Endocrinology*, **33**, 282–88 (1943)
55. Saier, E., Grauer, R. C., and Starkey, W. F., *J. Biol. Chem.*, **148**, 213–18 (1943)
56. Pincus, G., *Endocrinology*, **32**, 176–84 (1943)
57. Gallagher, T. F., and Munson, P. L. (Personal communication); Koch, F. C., and Hanke, M. E., *Practical Methods in Biochemistry*, 4th Ed., p. 280 (1943)
58. Szego, C. M., and Samuels, L. T., *J. Biol. Chem.*, **151**, 587–98 (1943)
59. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150**, 315–24 (1943)
60. Ruzicka, L., and Prelog, V., *Helv. Chim. Acta*, **26**, 975–95 (1943)
61. Ruzicka, L., Prelog, V., and Wieland, P., *Helv. Chim. Acta*, **26**, 2050–57 (1943)
62. Rosenheim, O., and Webster, T. A., *Biochem. J.*, **37**, 513–14 (1943)
63. Turfitt, G. E. *Biochem. J.*, **37**, 115–17 (1943)
64. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **149**, 505–9 (1943)
65. Bloch, K., Berg, B. N., and Rittenberg, D., *J. Biol. Chem.*, **149**, 511–17 (1943)
66. Schiller, S., and Dorfman, R. I., *Endocrinology*, **33**, 402–4 (1943)
67. Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, **148**, 455–56 (1943)
68. Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, **146**, 369–79 (1942)

69. Gallagher, T. F., Munson, P. M., and Koch, F. C., *Endocrinology*, **30**, S1036 (1942)
70. Talbot, N. B., Ryan, J., and Wolfe, J. K., *J. Biol. Chem.*, **148**, 593–602 (1943)
71. Talbot, N. B., Ryan, J. and Wolfe, J. K., *J. Biol. Chem.*, **151**, 607–14 (1943)
72. Woolf, R. B., Viergiver, E., and Allen, W. M., *J. Biol. Chem.*, **146**, 323–30 (1942)
73. Hoffman, M. M., Kazmin, V. E., and Browne, J. S. L., *J. Biol. Chem.*, **147**, 259–60 (1943)
74. Strickler, H. S., Shaffer, C. B., Wilson, D. A., and Strickler, E. W., *J. Biol. Chem.*, **148**, 251–52 (1943)
75. Pearlman, W. H., and Pincus, G., *J. Biol. Chem.*, **147**, 379–87 (1943)
76. Schiller, J., and Pincus, G., *Arch. Biochem.*, **2**, 317–21 (1943)
77. Pincus, G., and Pearlman, W. H., *Endocrinology*, **31**, 507–14 (1942)
78. Heller, C. G., and Heller, E. J., *Endocrinology*, **32**, 64–68 (1943)
79. Schiller, J., and Pincus, G., *Science*, **98**, 410–12 (1943)
80. Szego, C. M., and Samuels, L. T., *J. Biol. Chem.*, **151**, 599–605 (1943)
81. Cantarow, A., Paschkis, K. E., Rakoff, A. E., and Hansen, L. P., *Endocrinology*, **33**, 309–16 (1943)
82. Szego, C. M., and Barnes, R. H., *Endocrinology*, **32**, 367–68 (1943)
83. Biskind, G. R., and Meyer, M. A., *Proc. Soc. Exptl. Biol. Med.*, **53**, 91–94 (1943)
84. Zondek, B., Sulman, F., and Sklow, J., *Endocrinology*, **33**, 333–36 (1943)
85. Biskind, M. S., and Biskind, G. R., *Endocrinology*, **32**, 97–102 (1943)
86. Hill, B. R., and Longwell, B. B., *Endocrinology*, **32**, 319–26 (1943)
87. Hirschmann, H., *J. Biol. Chem.*, **150**, 363–79 (1943)
88. Werner, S. C., *J. Clin. Investigation*, **22**, 395–401 (1943)
89. Pincus, G., *J. Clin. Endocrinol.*, **3**, 195–99 (1943)
90. Pincus, G., and Hoagland, H., *J. Aviation Med.*, **14**, 3–23 (Aug., 1943)
91. Hollander, F., Kriss, B., Klempner, E., and Frank, R., *Endocrinology*, **33**, 217–23 (1943)
92. Nathanson, I. T., and Aub, J. C., *J. Clin. Endocrinol.*, **3**, 321–30 (1943)

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THE BIOCHEMISTRY OF THE NUCLEIC ACIDS, PURINES, AND PYRIMIDINES

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This review is concerned chiefly with the contributions which have appeared during the past three years on the biochemistry of nucleic acids and their hydrolysis products. The chemistry, function, and metabolism of the various adenine nucleotides and dinucleotides which have been included under the above title in other volumes of the *Annual Review of Biochemistry* have been largely omitted in the present report. These aspects of the subject have become an increasingly important part of the larger topic of intermediary carbohydrate metabolism and have recently been presented in *A Symposium on Respiratory Enzymes* (1). In a similar way, only certain phases of the biochemistry of uric acid have been included. Due to the war, a number of the papers mentioned have been available only in the form of abstracts. The author has attempted to present the results of such papers with the hope that they might be of interest even if a critical appraisal of the data is impossible at present.

Ribonucleic Acid

Molecular weight.—Since the discovery that four mononucleotides could be obtained by the mild hydrolysis of yeast ribonucleic acid, several different tetranucleotide formulas have been postulated for its structure (2, 3, 4). These have been made, however, largely without consideration as to whether or not the actual determination of molecular weight would lead to a value in agreement with the tetranucleotide theory. The first attempt to provide such correlation was that of Myrbäck & Jorpes (5), who showed by means of diffusion studies that yeast ribonucleic acid in the presence of appreciable amounts of sodium chloride gave diffusion constants[1] between 17 and 23 at 20°. Calculations of molecular weights by an empirical method gave values between 1,300 and 2,000. Under similar conditions, the ribonucleic acid of the pancreas gave diffusion constants between 12 and 16 and molecular weights between 2,700 and 5,200. While the method of calculating molecular weights was open to question, the results provided a

[1] Diffusion constant, $D_{20} \times 10^7$ cm^2 per sec.

range for the diffusion constants of the two ribonucleic acids and showed a surprising difference between them.

Diffusion studies on yeast ribonucleic acid have also been made by the author (6) and by Kunitz (7) and more recently on the ribonucleic acid of tobacco mosaic virus by Cohen & Stanley (8). Whereas commercial yeast ribonucleic acid gave values for the diffusion constant of the order of magnitude found by Myrbäck & Jorpes, purified preparations gave significantly lower values between 10 and 13 at 20°. Estimations of molecular weight from such data by the method used by Myrbäck & Jorpes or by means of Stokes' law give values several times that required by theory for the tetranucleotide structures mentioned above.

The experiments of Cohen & Stanley have established a polynucleotide structure for the ribonucleic acid of tobacco mosaic virus in an even more convincing fashion. Three types of virus ribonucleic acid preparations were compared by means of diffusion, sedimentation analysis, osmotic pressure, and viscosity measurements. Calculations of molecular weights and asymmetries of the different preparations showed that particle size and asymmetry were dependent on the method used in the preparation of the nucleic acid. That which gave the most asymmetric particle involved merely heat denaturation of the virus between pH 5 and 6 in the presence of sodium chloride. Such preparations were estimated to have molecular weights between 150,000 and 290,000 and to be highly asymmetric, having an axial ratio of 27 to 1. In the form of neutral gels they showed spontaneous birefringence. In contrast to these highly asymmetric preparations, those prepared by treatment with sodium hydroxide at 0° gave molecular weights of about 15,000, were less asymmetric (axial ratio of about 10 to 1), and failed to show spontaneous birefringence. It appears, therefore, that the native ribonucleic acid consists of highly asymmetric particles which either undergo spontaneous decomposition or are hydrolyzed by cold alkali to form a less-highly polymerized polynucleotide. An interesting paper with regard to the constituents of the ribonucleic acid of tobacco mosaic virus has appeared from Butenandt's laboratory (9). It was concluded from absorption spectrum measurements that, in the intact virus molecule, the purine and pyrimidine rings are oriented in approximately parallel planes perpendicular to the longitudinal axis of the nucleic acid molecule. It may be recalled that the same type of orientation was found from x-ray data for desoxyribonucleic acid by Astbury & Bell (10).

Complete studies similar to those carried out on the ribonucleic acid of tobacco mosaic virus have not been reported for that of yeast. In a recent paper, however, Tennent & Vilbrandt (11) have examined the sedimentation and diffusion rates of a sample of yeast ribonucleic acid prepared by Caspersson. The sedimentation constant could not be determined, but if the shape of the molecule were assumed to be the same as that of a partially-hydrolyzed sample of thymus nucleic acid, the molecular weight would be of the order of 4,000 to 5,000. As the history of this preparation was not published at the time, it is not known whether the sample had been freed from the lower molecular weight constituents which give a relatively high diffusion rate. As solutions of the purified commercial product show about the same specific viscosity as the alkali-treated virus nucleic acid, about 0.065 for a 1 per cent solution as dry nucleic acid in 0.2 *M* sodium borate at pH 7.8 (12), it seems likely that this material will prove comparable in molecular size to the tobacco mosaic virus nucleic acid prepared by alkali treatment.

The results of several other lines of investigation, however, can also be interpreted as consistent with the view that yeast ribonucleic acid consists of an asymmetric polynucleotide. Various investigators (13, 14, 15) have shown that purified yeast ribonucleic acid contains only four acid groups for each unit weight of about 1,286 (the molecular weight of a tetranucleotide). While this has been interpreted by Takahashi (4) and Makino (13) as evidence for a cyclic tetranucleotide [see also Gulland (14)], it is explained equally well by a relatively asymmetric, polynucleotide molecule, containing several tetranucleotide structures linked in a single chain. In such a structure the secondary phosphoric acid grouping postulated by Levene & Simms (3) can be considered linked to another tetranucleotide unit and the process repeated until a relatively asymmetric molecule is formed with only one terminal phosphoric acid group, being present as a monophosphoric ester. The simplest polynucleotide found by Cohen & Stanley for the alkali-treated virus ribonucleic acid would show, for example, 4.1 acid groups per tetranucleotide unit, a value in reasonable agreement with that mentioned above. Similarly, the more asymmetric polynucleotides should give a value even closer to four.

The data obtained by Gulland & Jackson (16) on the amount of inorganic phosphate liberated from yeast ribonucleic acid by phosphatase is also in agreement with the concept of a relatively asymmetric polynucleotide. As the terminal dibasic phosphate group of

such a molecule is the only one that should be split by phosphatase, the percentage of phosphate hydrolyzed should depend on the number of nucleotide units in the molecule. In the case of the tetranucleotide structure proposed by Levene & Simms, one should expect 25 per cent of the phosphorus to be hydrolyzed by phosphatase. Actually, the experiments of Gulland & Jackson show that usually the amount is much less than 7 per cent of the total nucleic acid phosphorus. If one phospho mono-ester group is present per molecule of polynucleotide of the size found by Cohen & Stanley for the alkali-treated virus nucleic acid, the amount of total phosphate hydrolyzed by phosphatase should be between 2 and 3 per cent.

Structure. — An important question relative to the structure of ribonucleic acid is whether or not guanine-uridylic acid occurs as a constituent along with the other recognized nucleotides. Further experiments have been reported by Bredereck, Berger & Richter (17) on its isolation from yeast ribonucleic acid. They find in agreement with the earlier results of Gulland and co-workers (18, 19) that only certain preparations of yeast ribonucleic acid give guanine-uridylic acid on hydrolysis. It is not clear, however, whether its occurrence depends on the method of preparation of the nucleic acid or on the type of yeast used. They again express the view that this compound is a secondary rather than a primary product of ribonucleic acid hydrolysis.

In experiments on the deamination of yeast ribonucleic acid with nitrous acid, they find that the original structure was preserved but that the aminopurines and aminopyrimidines were deaminated to the corresponding oxy compounds, which gave xanthine, hypoxanthine, and uracil after hydrolysis. The yield of deaminated ribonucleic acid was increased from 30 per cent in the earlier publication (20) to 47.3 per cent. The deaminated product gave a nitrogen to phosphorus ratio of 1.35 in agreement with the theory that each tetranucleotide unit contains one molecule each of guanine, adenine, and cytosine, and that the amino groups are uncombined.

In attempts to obtain products intermediate in size between mononucleotides and the original nucleic acid, the latter was subjected to hydrolysis in aqueous pyridine solution. In many cases it was possible to isolate guanylic acid as the brucine salt as well as a trinucleotide, which is stated to be homogeneous by analysis, titration, polarization, and by the quantitative determination of adenine. Further hydrolysis of the trinucleotide showed that adenylic acid was split off at the be-

ginning of the hydrolysis, whereas cytidylic and uridylic acids did not appear until later. This and the original isolation of guanylic acid is given as evidence for the occurrence of the purine nucleotides at the two ends of each tetranucleotide unit with the pyrimidine nucleotides occurring in the middle. Such results, however, were not found with all samples of ribonucleic acid used; in many instances, the other nucleotides were found along with guanylic acid after the initial hydrolysis.

A similar conclusion with regard to the relative positions of the purine and pyrimidine nucleotides in the tetranucleotide chain was reached by Bolomey & Allen (21) as the result of studies on the hydrolysis of yeast ribonucleic acid by crystalline ribonucleinase[2] and by a non-specific phosphatase. It was found that the purine nucleosides were liberated at faster rates than the pyrimidine nucleosides as determined by analyses for total purine nucleoside nitrogen, guanosine nitrogen, and inorganic phosphate. In the experiments with the non-specific phosphatase, the initial amounts of purine nucleoside formed were equivalent to the inorganic phosphate liberated. This is presented by the authors as definite proof that in this case no pyrimidine component was hydrolyzed. After a preliminary treatment with ribonucleinase, however, 0.38 equivalent of inorganic phosphate in excess of the amount required by the purine nucleosides found was liberated when the non-specific phosphatase was added. Because guanosine was produced at a faster rate than adenosine, it was suggested that guanylic acid occupies one of the outer positions in the tetranucleotide chain and that adenylic acid is either adjacent or at the other end of the chain. These conclusions, as well as those of Bredereck and co-workers, are based on the assumption of a tetranucleotide structure for the original ribonucleic acid and will require reinterpretation on the basis of the asymmetric polynucleotide structure discussed above as well as on new information which has been obtained as to the nature of ribonucleinase action (22).

Nature of ribonucleinase (ribonuclease) action.—Like many other phases of nucleic acid chemistry, the nature of ribonucleinase action has been a subject for controversy since the discovery of the enzyme by Jones in 1920 (23). Because four mononucleotides were isolated after enzymic hydrolysis, Jones & Perkins concluded that the action

[2] The name "ribonucleinase" is used for the crystalline enzyme instead of ribonuclease as provisionally suggested by Kunitz (7) because, as mentioned below, mononucleotides are produced as a result of ribonuclease action on ribonucleic acid.

of the enzyme consisted in breaking nucleotide linkages only (24). Levene, however, was not successful in his attempts to repeat these experiments (25, p. 312) and in a publication with Schmidt (26) reached the conclusion that "The function of the enzyme is that of a depolymerizing agent limited to the dissociation of the tetranucleotides of high molecular weight into those of lower molecular weight."

The nature of the split products formed as a result of ribonucleinase action was investigated by Loring & Carpenter (22) using highly purified crystalline preparations of ribonucleinase. Application of the general fractionation procedure used for the preparation of mononucleotides to enzyme-treated yeast ribonucleic acid resulted in the isolation of the four mononucleotides, either in their free form or as well-characterized salts. Control experiments on ribonucleic acid, in the absence of enzyme treatment, showed that the fractionation procedures alone did not lead to the formation of mononucleotides. The original finding of Jones & Perkins has thus been confirmed with the crystalline enzyme. In conformity with the nomenclature proposed by Levene & Medigreceanu (27), it was suggested that the crystalline enzyme, provisionally named ribonuclease by Kunitz (7), be called instead ribonucleinase. In view of the fact that the four mononucleotides are produced as a result of ribonucleinase action, it appears to the writer that the results of Bolomey & Allen can be interpreted equally well by differences in the rates at which the different nucleotides are hydrolyzed by the non-specific phosphatase.

Studies on the nature of ribonucleinase action have led to a further important contribution to the chemistry of ribonucleic acid. Kunitz showed that whereas a portion of the treated nucleic acid was easily diffusible through cellophane, from 40 to 50 per cent was relatively non-diffusible. Similarly, only about 40 per cent of the original nucleic acid phosphorus was rendered soluble in uranium acetate-trichloroacetic acid solution after ribonucleinase treatment. This observation has been confirmed and extended in the writer's laboratory (28). It has been found that even in the presence of a large excess of enzyme or under conditions in which the easily diffusible products are removed, a relatively large proportion of the original nucleic acid is resistant to further enzyme action. It is evident, therefore, that yeast ribonucleic acid must contain at least two different types of linkages, one of which is labile and one of which is resistant to the action of ribonucleinase. Whether or not these results can be correlated with those of Gulland and co-workers (16), which show that

only from 35 to 75 per cent of the phosphorus of ribonucleic acid is liberated by the action of various mixtures of monoesterases and diesterases, remains for further study.

Desoxyribonucleic Acids

Physico-chemical studies.—Further studies on the physico-chemical properties of desoxyribonucleic acid have appeared from several laboratories. Pedersen, in a brief report (29), gives a value of about 200,000 for the average molecular weight of desoxyribonucleic acid prepared by Hammarsten's method. This value is somewhat lower than that found originally by Signer, Caspersson & Hammarsten, i.e., 500,000 to 1,000,000 (30). The sedimentation rate was dependent on concentration and the frictional ratio, f/fo, was 2.5. As reported by other workers, this material was not monodisperse. Tennent & Vilbrandt (11) have examined the sedimentation and diffusion rates of five preparations of sodium thymonucleate and have calculated molecular weights. They find the most highly polymerized products to have molecular weights of the order of 500,000 and the greatly degraded samples about 5,000.

In an attempt to characterize desoxyribonucleic acid more fully, Greenstein & Jenrette (31, 32) have studied the physical changes produced in the molecule by various types of treatment. Preparations were made by the Feulgen-Levene procedure (25, p. 295) and by that of Hammarsten (33). A comparison of the two showed that the streaming birefringence and viscosity of solutions of the latter were from three to five times as great as those of the former. Addition of salts to solutions of sodium desoxyribonucleate resulted in a decrease and finally in the complete disappearance of streaming birefringence. Considerable differences in the ability of different salts to effect this change were observed, with salts of guanidine being particularly effective. Of striking interest was the observation that the phenomenon was completely reversible. When the salt was removed as by dialysis, the solutions of nucleic acid regained completely their property of showing streaming birefringence. Amino acids showed a similar effect to that of salts, and purified proteins such as crystalline egg albumin and horse serum albumin on a molar basis were many times as active as the same concentration of salts or amino acids. When extracts of tumor or normal tissue or milk or sera of various species were added to the solutions of sodium desoxyribonucleate, it was found that in addition to the immediate initial drop in viscosity and streaming bi-

refringence due to the presence of inert proteins, amino acids, and salts, there was a further decrease due to the presence of a depolymerizing factor, which has been called thymonucleodepolymerase. Whereas the original nucleic acid solutions show the property of anomalous or structural viscosity, this was entirely lost after treatment with the depolymerase. It is suggested that the thymonucleate at the latter stage was completely or nearly completely depolymerized.

In a later paper (34) the former author showed that the colloid osmotic pressure of solutions of desoxyribonucleic acid decreased to practically zero when serum or serum albumin was added to the solution. The explanation was tentatively offered that the decrease in osmotic pressure as well as the decrease in streaming birefringence and viscosity under these conditions is due to a suppression of dissociation of the nucleate, probably accompanied by aggregation.

Nucleoproteins of the cell.—Of particullar interest has been the report by Mirsky & Pollister (35, 36) on the preparation of fibrous nucleoproteins from cell nuclei. It was shown by these authors that when minced tissue, that had been washed with physiological saline, was extracted with 1 *M* sodium chloride, a viscous solution was obtained. When the latter was added to six volumes of water, a fibrous mass of nucleoprotein separated which could be redissolved in 1 *M* sodium chloride and reprecipitated as before. Solutions of these nucleoproteins show a striking viscosity and birefringence of flow. Highly polymerized desoxyribonucleic acid was readily obtained by dialysis of the nucleoprotein solutions against 1 *M* sodium chloride, for under these conditions the protamine gradually diffused through the membrane leaving the nucleic acid behind. In this way fibrous nucleoproteins and in some cases desoxyribonucleic acid were obtained from mammalian liver, kidney, spleen, brain, pancreas, thymus; from frog, trout, shad, and sea urchin sperm; from the liver, spleen, and blood cells of the dog fish; and from wheat germ.

A somewhat different method for the preparation of cell nucleoproteins is that used by Mayer & Gulick (37). In this case the denser nuclear material was separated from other cell constituents by sedimentation in an inert liquid of intermediate specific gravity. Evidence was obtained for the presence of appreciable amounts of nucleic acid-free protein as well as nucleoprotein in their preparations.

Relation to cell nuclei.—Through the work of a number of different investigators, methods are now available for the preparation of cell nuclei on a relatively large scale [Crossmon (38), Stoneburg (39),

Marshak (40), Laskowski (41), Lazarow (42)]. Several workers have studied the enzyme systems of such preparations [Lardy & Phillips (43), Zittle & Zitin (44), Dounce (45)]. Dounce (45) has examined samples of rat liver nuclei for several different enzymes. Arginase, cytochrome oxidase, esterase, lactic acid dehydrogenase, alkaline phosphatase, and acid phosphatase were found in fairly high concentrations. Catalase, succinic acid dehydrogenase, cytochrome-*c*, and coenzyme I were either absent or present in low concentration.

Conflicting reports have been presented as to the relative amounts of desoxyribonucleic acid in malignant as compared to normal tissue. Vowles (46), by means of the diphenylamine reaction of Dische, found about the same concentration in samples of Jensen sarcoma as in liver, spleen, and kidney. A different result was reported in cancer produced in rat liver by the feeding of butter yellow (47). After from 17 to 59 days, the thymonucleic acid content of the liver almost doubled and in the cancerous portion the concentration was even higher. A similar result was obtained with tumor cells by Koller (48), who believes that the nucleic acid content of chromosomes rises to abnormally high values in such cases. Dounce (49), however, has analyzed samples of Walker carcinosarcoma and hepatoma and has found about the same concentration of desoxyribonucleic acid as was present in normal liver tissue. Nuclei of bird erythrocytes and fish spermatozoa, however, contained a much higher desoxyribonucleic acid content. The differences as far as malignant tissue is concerned may no doubt be resolved when the same kind of tissue is examined under comparable conditions as to age, rate of growth, etc. It is evident, however, that a general statement as to the relative nucleic acid concentration in malignant tissue cannot be made.

A somewhat different observation obtained by ultraviolet spectroscopy is that of Caspersson *et al.* (50) that rapidly growing malignant cells like normal ones contain ribose nucleotides as well as desoxyribonucleic acid.

Structure.—In a paper on the constitution of thymonucleic acid, Bredereck *et al.* (51) have subjected the latter to methylation with dimethyl sulfate. Analyses of the once methylated product showed the presence of two methoxy and seven N-methyl groups for each four phosphorus atoms. After a second methylation treatment the number of methoxy groups increased to three. Hydrolysis of the methylated nucleic acid with methyl alcohol and hydrochloric acid gave 1,6-dimethyladenine, which was isolated as the picrate. Hydrolysis with

25 per cent sulfuric acid at 180° gave 1,6-dimethylcytosine and 1-methylthymine. Methylation and hydrolysis of guanosine, adenosine, and cytidine by similar procedures gave respectively dimethylguanosine, dimethyladenine, and dimethylcytosine, which in the last two instances were identical with the products obtained from the methylated desoxyribonucleic acid. The fact that methylation in the original nucleic acid or in the ribonucleosides occurs at the 1- and 6- positions in both adenine and cytosine and at the 1- position in thymine is given as proof that these positions are unsubstituted and that the linkage between sugar and adenine must therefore be in the 9- position. Similarly, the linkage between sugar and cytosine and sugar and thymine must be in the 3- position. These experiments confirm similar conclusions of other workers with respect to the pyrimidine nucleosides (52) and of Gulland and co-workers (53) for the purine nucleosides.

Electrophoresis

Electrophoretic studies on ribonucleic and desoxyribonucleic acids have been reported by several investigators (54 to 56a). In general, the results are in agreement that the various samples show a high mobility of the order of 15 to 23.5×10^{-5} cm. per sec. per volt per cm. at pH values between 6 and 7.9 and are electrophoretically homogeneous. There has been disagreement, however, as to whether the two types show the same or different mobilities. The results of Hall (54) on thymonucleic acid of the Hammarsten type are in essential agreement with those of Stenhagen & Teorell (55) if allowances are made for differences in the ionic strengths of the buffer solutions used. In contrast to the more recent titration data on ribo- and desoxyribonucleic acid which indicate four and five acidic groups respectively per unit weight of about 1,300, the electrophoretic results of Zittle & Siebert (56) show the same net charge for the two types as they occur in certain preparations from hemolytic streptococci. Cohen, however, has compared the mobilities of Hammarsten desoxyribonucleic and of ribonucleic acids and finds the former to migrate with a mobility about 21 per cent greater than that of the latter (56a). After treatment of the latter with ribonucleinase, the mobility was increased about 21 per cent as would be expected because of the presence of the more acidic mononucleotides (two acidic groups per mol) as discussed above.

In a paper on the electrophoretic behavior of mixtures of egg albumin and nucleic acid on either side of and at the isoelectric point of

the protein, Longsworth & MacInnis show that the components migrate independently on the alkaline side (57). On the acid side and at the isoelectric point, however, the electrophoretic data reveal the presence of a dissociable complex. Similarly, Zittle & Siebert (56) have shown that the nucleic acid present in preparations of the type specific M-protein behaves as a separate component electrophoretically.

Nucleotides and Nucleosides

Uridylic acid and guanosine.—The synthesis of uridylic acid by the condensation of trityl uridine with diphenylphosphoryl chloride at $-18°$ in pyridine solution and hydrolysis of the product with sodium hydroxide was reported by Bredereck & Berger (58). In the same paper, several new derivatives of guanosine were also described, namely 3,5-benzylideneguanosine, acetylbenzylideneguanosine, acetylguanosine, acetyltritylguanosine, and tritylguanosine.

Adenylic acid.—Buell has developed a new method for the preparation of either crystalline adenosine-3-phosphate or adenosine-5-phosphate which appears advantageous for the recovery of these compounds in pure form (59). The nucleotide is precipitated from its solutions as an insoluble aluminum picrate complex. Yields of 1.19 gm. of crystalline adenosine-3-phosphate from 20 gm. of yeast nucleic acid and of 800 mg. of adenosine-5-phosphate from eleven pounds of heart muscle were reported.

Another method of interest for the preparation of adenosine-5-phosphate is that of Kerr (60) in which adenosinetriphosphate was split by alkaline hydrolysis. A yield of about 72 per cent of the theoretical was reported. An improved method for the preparation of the triphosphate from muscle (61) and its isolation from brain (62) were also reported.

Adenosine-3-phosphate has been synthesized by Barker & Gulland (63) from adenosine and phosphorus oxychloride. The identity of the synthetic product was established by determination of its optical activity and by its resistance to dephosphorylation with Russel's viper venom, which had formerly been shown to contain only a 5-nucleotidase, an enzyme which specifically dephosphorylates adenosine-5-phosphate (64).

Preparation of nucleosides.—Another paper has appeared from Bredereck's laboratory on the preparation of adenosine, guanosine, cytidine, and uridine after hydrolysis of yeast ribonucleic acid either

by a sweet almond enzyme extract or by boiling aqueous pyridine (65). Yields from 100 gm. of nucleic acid by either of the two procedures were reported as follows: guanosine, 20 to 25 gm.; adenosine, 10 to 14 gm.; cytidine sulfate, 8 to 10 gm.; and uridine, 2 to 3 gm.

The precipitation of nucleotides, nucleosides, purines, or pyrimidines by various metallic salts has been examined by Inagaki (66). Of those studied, mercuric nitrate was found most satisfactory for the precipitation of all nitrogen-containing substances of these types.

A highly active preparation of intestinal nucleotidase which dephosphorylates about 300 times its weight of ribonucleotides in twelve hours was described by Lehmann-Echternacht (67). The product was inactive as a polynucleotidase and as a nucleosidase but showed both 3-phospho, 5-phospho, and pyrophosphatase activity. Schmidt & Thannhauser (68) found that intestinal phosphatase, unlike ribonucleinase of the pancreas, was destroyed when the extract at pH 5 was heated to 80° for fifteen minutes. Thymonucleic acid was not hydrolyzed by the enzyme.

Desoxyribo-nucleotides and nucleosides.—A series of papers dealing with various enzyme preparations which hydrolyze desoxyribonucleic acid and its nucleotides has been published by Lehmann-Echternacht (69) and Fischer *et al.* (70). Three types of enzyme preparations were described: (*a*) a polynucleotidase which hydrolyzes the nucleic acid to a product which is regarded as a tetranucleotide and is called oligonucleotide, (*b*) a desriboligonucleotide which converts oligonucleotide to nucleotides, and (*c*) a nucleotidase which liberates phosphate. The oligonucleotide, unlike the original nucleic acid, was no longer precipitated by hydrochloric acid. It formed salts with copper, silver, cadmium, mercury, lead, and bismuth that were more soluble than the corresponding ones of the original nucleic acid. In the hydrolysis of the oligonucleotide it was found that thymine- and adenine-desoxyribosides were first liberated. It is therefore suggested that these nucleotides occupy the external positions in the tetranucleotide chain. It appears to the writer that conclusions regarding the structure of desoxyribonucleic acid based on results obtained with impure enzyme preparations are highly inconclusive.

Bredereck *et al.* have also proposed (71) that the product formerly described as thymonucleic acid (20) probably has a tetranucleotide structure. Values for the molecular weight between 1,196 and 1,274 were found as compared to the theoretical of 1,309.

A modification of the method of Klein & Thannhauser (72) for

the preparation of adenine-desoxyriboside has been published by Brady (73). The crystalline product melted at 187–188° instead of at 181° as previously reported. The author suggests that the new preparation which agreed in solubility, crystalline form, and specific rotation with that of Klein probably represents a purer sample. The preparation of thymine-desoxyriboside with the same properties as reported by Klein was also confirmed.

METABOLISM

During the past three years the problem of the metabolism of purines and pyrimidines has been attacked with radioactive phosphorus and with isotopic nitrogen. The slow but appreciable turnover of nucleic acid phosphorus in rabbit tissues after administration of isotopic phosphorus and the rapid turnover in other organic phosphorus fractions found by Hahn & Hevesy (74) have been confirmed by Brues *et al.* (75). Injection of radioactive phosphorus, P^{32}, as neutral phosphate into rats and subsequent examination of various liver fractions showed that the turnover of phosphorus in the nucleic acid portion was less than that in the phospholipid, "total protein," or "residual protein" fractions. In regenerating liver there was an increased P^{32} uptake in the nucleic acid fraction, which could be accounted for by the synthesis of nucleic acid in the formation of new cells. The discrepancy between these observations and those of Marshak on isolated nuclei (40), those of Tuttle *et al.* (76) on leukemic infiltrated mouse tissue, and those of Kohman & Rusch with liver carcinoma (77) are explained in the paper by Brues *et al.* by the rapid turnover of phosphorus in the "total protein" or "nucleoprotein" fraction. In the previous experiments, the latter had not been distinguished from the nucleic acid fraction.

In extensive experiments, Barnes & Schoenheimer (78, 79) have studied the distribution of the nitrogen isotope, N^{15}, in the excreta and in the internal organs of pigeons and rats that were fed N^{15} ammonia or N^{15} urea. The results with pigeons showed that ammonia nitrogen was rapidly incorporated into the uric acid excreted and to a lesser extent into the mixed purines of the internal organs. Isolation of guanine, adenine, cytosine, and thymine from the nucleic acid fraction showed about the same concentration of N^{15} in each of these constituents. After the feeding of N^{15} urea to pigeons, however, only small amounts of the isotope were found in either the mixed purines or the uric acid excreted. Examination of isotope concentration in the xan-

thine and hypoxanthine obtained from guanine and adenine respectively showed that N^{15} was present in both cases in the purine rings as well as in the amino groups. Similarly, about the same concentration of N^{15} was found in thymine, which contains no amino group, as in cytosine which does. In contrast to the adenine present in nucleic acid, however, only about one fourth to one third as much isotope nitrogen was found in the adenylic acid isolated from pigeon breast muscle.

When isotopic ammonia was fed to rats, similar results were obtained in that N^{15} was found in the purines and pyrimidines obtained from the nucleic acid fraction and in the nitrogen present in the ring as well as in substituent amino groups. In agreement with the concept that allantoin is the end-product of purine metabolism in the rat, about the same isotope concentration was found in the allantoin excreted as in the purines.

The experiments in pigeons have therefore confirmed others which showed that uric acid in birds is formed either from ammonia or from amino acids (80). They also show that urea, arginine, and histidine are not normal intermediates in purine or pyrimidine synthesis in either pigeons or rats. The evidence also indicates that the purines and pyrimidines of nucleic acids, like the amino acids in proteins, exist in a state of "dynamic equilibrium" with a generally available nitrogen "pool" and that nitrogen replacement takes place continuously in the purine and pyrimidine rings as well as in the substituent amino groups. The results with muscle adenylic acid suggest that the adenine present in this compound must follow a somewhat different metabolic pathway than that of nucleic acid.

In dogs, unlike rats, histidine appears to function as an intermediate in allantoin synthesis. For as Popiel has shown (81), the injection of this amino acid into the portal vein leads to a large increase in the allantoin content of the liver.

The following are a few miscellaneous observations which also have some bearing on purine and pyrimidine metabolisms. Vilter and co-workers (82) have shown that the administration of adenylic acid to patients with a nutritional deficiency disease resulted in definite clinical improvement. Intravenous injection in doses of 50 mg. led to the disappearance of ulcers in the mouth and to rapid improvement in pellagrous glossitis or subclinical pellagra. Some individuals failed to respond to treatment with brewers' yeast or thiamin hydrochloride but were helped when yeast adenylic acid was administered.

A further relationship between thiamin and adenylic acid is suggested by the work of Kühnau & Schiering (83), who found that the blood of patients with gout contained more nucleotides than that of normal individuals. The symptoms in this case were alleviated by treatment with thiamin hydrochloride.

Barrenscheen & Peham (84) have examined various tissues, including pancreas, kidney, brain, spleen, and thymus, for their purine nucleotide, nucleoside, and free purine content. An insignificant amount of free purine was found in fresh muscle. The purine content of the various tissues, as nucleic acid, was essentially less than the total purine content, varying from 10 per cent in striped muscle to about 25 per cent in kidney, brain, liver, spleen, and pancreas. The latter observation shows that by far the greater proportion of the total purine is present as nucleotide or nucleoside.

Purine and Pyrimidine Compounds as Growth Factors

Since the observation of Richardson in 1936 (85) that uracil was necessary for the growth of *Staphylococcus aureus* under certain conditions, many reports have appeared dealing with the requirements of a number of microorganisms for one or another of the various hydrolytic products of nucleic acid. Möller showed that adenine was required for the growth of *Streptobacterium plantarum* (86). Pappenheimer & Hottle found that adenine or hypoxanthine, guanine, xanthine, guanylic acid, or adenylic acid was required for the growth of a strain of Group A hemolytic streptococci (87). Snell & Mitchell have examined the growth requirements of *Lactobacillus arabinosus, Lactobacillus pentosus, Leuconostoc mesenteroides,* and *Streptococcus lactis* R. (88). They found that under certain conditions each of the purine or pyrimidine bases of nucleic acid could become the limiting factor for growth. Adenine greatly stimulated the growth of *L. arabinosus* and *L. pentosus* and was essential for the growth of *S. lactis,* as was also thymine. Uracil was helpful for the growth of *L. arabinosus* and *L. mesenteroides,* but guanine was essential in the latter instance. Similarly, although the compound obtained from solubilized liver by Stokstad (89) was more active in providing for growth of *Lactobacillus casei,* it could be replaced by guanine, adenine, hypoxanthine, xanthine, and thymine but not by uracil or cytosine.

More recently, Feeney & Strong (90) have shown that the yeast extract which has been required for the growth of *Lactobacillus casei* may be replaced by a mixture of known compounds including adenine

and guanine. A somewhat different result is that of Pennington who has studied the growth requirements of *Spirillum serpens* (91). Guanine and adenine when tested singly had no activity but hypoxanthine provided satisfactory growth. Uric acid, xanthine, uracil, adenosine, or yeast adenylic acid showed no activity. Robbins & Kavanagh have reported that the growth factor for *Phycomyces,* factor Z_1, can be replaced by guanine (92) and by hypoxanthine (93). The latter compound was isolated as the silver salt from extracts of potato tubers in amounts which accounted for the factor Z_1 activity of the extracts. Various related substances including isoguanine, xanthine, adenine, 2-aminouric acid, theobromine, theophyllin, and guanosine were inactive.

In several instances mentioned above, purine nucleosides or nucleotides were found as effective as the free purine itself. In no case in which pyrimidines were found effective was the activity of either the corresponding pyrimidine nucleoside or nucleotide determined. Such studies have recently been made by Loring & Pierce (94) on two experimentally produced, pyrimidine-deficient *Neurospora* mutants. Whereas good growth was obtained in the presence of uracil, the corresponding ribonucleoside and ribonucleotide, uridine and uridylic acid, were from ten to sixty times as active as the free pyrimidine. Even more striking results were obtained in the case of cytosine for here the free pyrimidine was completely ineffective for growth whereas cytidine and cytidylic acid were highly active. It seems possible that a similar situation may be found in the cases mentioned above in which free pyrimidines have been shown to have activity as growth factors. These experiments on the availability of the pyrimidine nucleotides and nucleosides for the growth of microorganisms take on added significance when it is recalled that Mendel & Myers (95) and Emerson & Cerecedo (96) found uracil and cytosine to be excreted largely as the free pyrimidines when they were fed to dogs; nucleic acid (95) or the pyrimidine nucleotides or nucleosides (96), on the contrary, were readily metabolized. It should be pointed out as well that no definite evidence has been presented for the occurrence of a pyrimidine ribonucleosidase.

Whereas the exact function of purines and pyrimidines as growth factors of microorganisms is not known, it has been shown by McIlwain (97) in the case of *Bact. coli* and *Streptococcus hemolyticus* that the metabolic processes blocked by acriflavin (a mixture of 2,7-diaminoacridine, proflavine, and euflavine) are reconstituted if nucleic

acid or nucleotides are supplied. It appears therefore that the bacteriostatic action of acriflavin is concerned with nucleic acid metabolism. These results are highly significant in providing another example of an agent which acts to prevent antibacterial action.

LITERATURE CITED

1. VARIOUS AUTHORS, *A Symposium on Respiratory Enzymes* (University of Wisconsin Press, Madison, 1942)
2. FEULGEN, R., *Z. physiol. Chem.*, **101**, 288 (1918)
3. LEVENE, P. A., AND SIMMS, H. S., *J. Biol. Chem.*, **70**, 327 (1926)
4. TAKAHASHI, H., *J. Biochem. (Japan)*, **16**, 463 (1932)
5. MYRBÄCK, K., AND JORPES, E., *Z. physiol. Chem.*, **237**, 159 (1935)
6. LORING, H. S., *J. Biol. Chem.*, **128**, lxi (1939)
7. KUNITZ, M., *J. Gen. Physiol.*, **24**, 15 (1940)
8. COHEN, S. S., AND STANLEY, W. M., *J. Biol. Chem.*, **144**, 589 (1942)
9. BUTENANDT, A., FRIEDRICH-FREKSA, H., HARTWIG, S., AND SCHEIBE, G., *Z. physiol. Chem.*, **274**, 276 (1942)
10. ASTBURY, W. T., AND BELL, F. O., *Nature*, **141**, 747 (1938)
11. TENNENT, H. G., AND VILBRANDT, C. F., *J. Am. Chem. Soc.*, **65**, 424 (1943)
12. LORING, H. S. (Unpublished data)
13. MAKINO, K., *Z. physiol. Chem.*, **232**, 229 (1935)
14. GULLAND, J. M., *J. Chem. Soc.*, 1722 (1938)
15. ALLEN, F. W., AND EILER, J. J., *J. Biol. Chem.*, **137**, 757 (1941)
16. GULLAND, J. M., AND JACKSON, E. M., *J. Chem. Soc.*, 1492 (1938)
17. BREDERECK, H., BERGER, E., AND RICHTER, F., *Ber. deut. chem. Ges.*, **74**, 338 (1941)
18. FALCONER, R., GULLAND, J. M., HOBDAY, G. I., AND JACKSON, E. M., *J. Chem. Soc.*, 907 (1939)
19. GULLAND, J. M., *Chemistry & Industry*, **59**, 321 (1940)
20. BREDERECK, H., KÖTHNIG, M., AND LEHMANN, G., *Ber. deut. chem. Ges.*, **71**, 2613 (1938)
21. BOLOMEY, R. A., AND ALLEN, F. W., *J. Biol. Chem.*, **144**, 113 (1942)
22. LORING, H. S., AND CARPENTER, F. H., *J. Biol. Chem.*, **150**, 381 (1943)
23. JONES, W., *Am. J. Physiol.*, **52**, 203 (1920)
24. JONES, W., AND PERKINS, M. E., *J. Biol. Chem.*, **55**, 557 (1923)
25. LEVENE, P. A., AND BASS, L. W., "Nucleic acids," *Am. Chem. Soc.*, Monograph Ser. (New York, 1931)
26. SCHMIDT, G., AND LEVENE, P. A., *J. Biol. Chem.*, **126**, 423 (1938)
27. LEVENE, P. A., AND MEDIGRECEANU, F., *J. Biol. Chem.*, **9**, 389 (1911)
28. CARPENTER, F. H., ROLL, P. M., AND LORING, H. S. (Unpublished data)

29. Svedberg, T., and Pedersen, K. O., *The Ultracentrifuge,* p. 433 (Oxford University Press, Oxford, 1940)
30. Signer, R., Caspersson, T., and Hammarsten, E., *Nature,* **141,** 122 (1938)
31. Greenstein, J. P., and Jenrette, W. V., *J. Natl. Cancer Inst.,* **1,** 77, 91 (1940)
32. Greenstein, J. P., and Jenrette, W. V., *Cold Spring Harbor Symposia Quant. Biol.,* **9,** 236 (1941)
33. Hammarsten, E., *Biochem. Z.,* **144,** 383 (1924)
34. Greenstein, J. P., *J. Biol. Chem.,* **150,** 107 (1943)
35. Mirsky, A. E., and Pollister, A. W., *Proc. Natl. Acad. Sci. U.S.,* **28,** 344 (1942)
36. Mirsky, A. E., *Advances in Enzymology,* **3,** 1 (1943)
37. Mayer, D. T., and Gulick, A., *J. Biol. Chem.,* **146,** 433 (1942)
38. Crossmon, G., *Science,* **85,** 250 (1937)
39. Stoneburg, C. A., *J. Biol. Chem.,* **129,** 189 (1939)
40. Marshak, A., *J. Gen. Physiol.,* **25,** 275 (1941)
41. Laskowski, M., *Proc. Soc. Exptl. Biol. Med.,* **49,** 354 (1942)
42. Lazarow, A., *J. Biol. Chem.,* **140,** p. lxxv (1941)
43. Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.,* **138,** 195 (1941)
44. Zittle, C. A., and Zitin, B., *J. Biol. Chem.,* **144,** 99 (1942)
45. Dounce, A. L., *J. Biol. Chem.,* **147,** 685 (1943)
46. Vowles, R. B., *Arkiv. Kemi, Mineral. Geol.,* **14 B,** 5 (1940); *Chem. Abstracts,* **35,** 1856 (1941)
47. Masayama, T., and Yokoyama, T., *Gann.,* **34,** 174 (1940); *Chem. Abstracts,* **35,** 3319 (1941)
48. Koller, P. C., *Nature,* **151,** 244 (1943)
49. Dounce, A. L., *J. Biol. Chem.,* **151,** 235 (1943)
50. Caspersson, T., Nyström, C., and Santesson, L., *Naturwissenschaften,* **29,** 29 (1941); *Chem. Abstracts,* **35,** 7516 (1941)
51. Bredereck, H., Müller, G., and Berger, E., *Ber. deut. chem. Ges.,* **73,** 1058 (1940)
52. Levene, P. A., and Tipson, R. S., *J. Biol. Chem.,* **104,** 385 (1934)
53. Gulland, J. M., and Story, L. F., *J. Chem. Soc.,* 692 (1938); Gulland, J. M., and Holiday, E. R., *J. Chem. Soc.,* 765 (1936)
54. Hall, J. L., *J. Am. Chem. Soc.,* **63,** 794 (1941)
55. Stenhagen, E., and Teorell, T., *Trans. Faraday Soc.,* **35,** 743 (1939)
56. Zittle, C. A., and Siebert, F. B., *J. Immunol.,* **43,** 47 (1942)
56a. Cohen, S. S., *J. Biol. Chem.,* **146,** 471 (1942)
57. Longsworth, L. G., and MacInnis, D. A., *J. Gen. Physiol.,* **25,** 507 (1942)
58. Bredereck, H., and Berger, E., *Ber. deut. chem. Ges.,* **73,** 1124 (1940)
59. Buell, M. V., *J. Biol. Chem.,* **150,** 389 (1943)

60. KERR, S. E., *J. Biol. Chem.*, **139**, 131 (1941)

61. KERR, S. E., *J. Biol. Chem.*, **139**, 121 (1941)

62. KERR, S. E., *J. Biol. Chem.*, **140**, 77 (1941)

63. BARKER, G. R., AND GULLAND, J. M., *J. Chem. Soc.*, 231 (1942)

64. GULLAND, J. M., AND JACKSON, E. M., *Biochem. J.*, **32**, 597 (1938)

65. BREDERECK, H., MARTINI, A., AND RICHTER, F., *Ber. deut. chem. Ges.*, **74**, 694 (1941)

66. INAGAKI, T., *J. Biochem. (Japan)*, **32**, 63 (1940); *Chem. Abstracts*, **35**, 762 (1941)

67. LEHMANN-ECHTERNACHT, H., *Z. physiol. Chem.*, **269**, 169 (1941); *Chem. Abstracts*, **36**, 6213 (1942)

68. SCHMIDT, G., AND THANNHAUSER, S. J., *J. Biol. Chem.*, **149**, 369 (1943)

69. LEHMANN-ECHTERNACHT, H., *Z. physiol. Chem.*, **269**, 201 (1941); *Chem. Abstracts*, **36**, 6552 (1942)

70. FISCHER, F. G., LEHMANN-ECHTERNACHT, H., AND BÖTTGER, I., *J. prakt. Chem.*, **158**, 79 (1941); *Chem. Abstracts*, **36**, 785 (1942)

71. BREDERECK, H., AND JOCHMANN, I., *Ber. deut. chem. Ges.*, **75**, 395 (1942); *Chem. Abstracts*, **37**, 3102 (1943)

72. KLEIN, W., AND THANNHAUSER, S. J., *Z. physiol. Chem.*, **218**, 173 (1933)

73. BRADY, T. G., *Biochem. J.*, **35**, 855 (1941)

74. HAHN, L., AND HEVESY, G., *Nature*, **145**, 549 (1940)

75. BRUES, A. M., TRACY, M. M., AND COHN, W. E., *Science*, **95**, 558 (1942)

76. TUTTLE, L. E., ERF, L. A., AND LAWRENCE, J. H., *J. Clin. Investigation*, **20**, 57 (1941)

77. KOHMAN, T. P., AND RUSCH, H. P., *Proc. Soc. Exptl. Biol. Med.*, **46**, 403 (1941)

78. SCHOENHEIMER, R., *The Dynamic State of Body Constituents*, 78 pp. (Harvard University Press, Cambridge, 1942)

79. BARNES, F. W., JR., AND SCHOENHEIMER, R., *J. Biol. Chem.*, **151**, 123 (1943)

80. SCHULER, W., AND REINDEL, W., *Z. physiol. Chem.*, **234**, 63 (1935)

81. POPIEL, L. V., *J. Physiol. (U.S.S.R.)*, **29**, 362 (1940)

82. VILTER, R. W., BEAN, W. B., AND SPIES, T. D., *J. Lab. Clin. Med.*, **27**, 527 (1942)

83. KÜHNAU, J., AND SCHIERING, M., *Klin. Wochschr.*, **19**, 705 (1940)

84. BARRENSCHEEN, H. K., AND PEHAM, A., *Z. physiol. Chem.*, **272**, 87 (1942)

85. RICHARDSON, G. M., *Biochem. J.*, **30**, 2185 (1936)

86. MÖLLER, E. F., *Z. physiol. Chem.*, **260**, 246 (1939)

87. PAPPENHEIMER, A. M., JR., AND HOTTLE, G. A., *Proc. Soc. Exptl. Biol. Med.*, **44**, 645 (1940)

88. SNELL, E. E., AND MITCHELL, H. K., *Proc. Natl. Acad. Sci. U.S.*, **27**, 1 (1941)

89. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475 (1941)
90. Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, **64**, 881 (1942)
91. Pennington, D. E., *Proc. Natl. Acad. Sci. U.S.*, **28**, 272 (1942)
92. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.*, **28**, 4 (1942)
93. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.*, **28**, 65 (1942)
94. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.* (In press)
95. Mendel, L. B., and Myers, V. C., *Am. J. Physiol.*, **26**, 77 (1910)
96. Emerson, O. H., and Cerecedo, L. R., *J. Biol. Chem.*, **87**, 453 (1930)
97. McIlwain, H., *Biochem. J.*, **35**, 1311 (1941)

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MINERAL METABOLISM

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The classification of physiology and biochemistry into compartments is becoming more and more unreal and artificial. Every aspect of mineral metabolism, for instance, demands an appreciation of the niceties of organic change, for the life of the cell depends upon the intimate association of its organic and inorganic elements. Mineral metabolism, however, has acquired a conventional meaning, and the annual review of it has come to imply a discussion of investigations in which some mineral element has played a more or less prominent part. Lately, the war has limited the scope of such reviews, for journals published in enemy and occupied countries are not coming to hand, and many of the reports in Britain and the United States are not being released for publication. Within the sense of these conventions and limitations, however, we have endeavoured to present a reasonably balanced picture of the expansion of our knowledge of the mineral exchanges of the body within the last twelve to eighteen months.

Our knowledge of the laws which govern the absorption and excretion of minerals has expanded considerably during recent years, and some general principles have now become firmly established. One is that minerals are much more readily absorbed if they are in solution, and consequently any ion which precipitates another in the stomach or intestine will, *ipso facto,* prevent its absorption. Soluble aluminum salts, for example, will inevitably react with phosphates and precipitate them irreversibly in the gut (1, 2). Competition for an insoluble anion, moreover, can be invoked to explain the effects of administering one metal, e.g., iron, upon the absorption of another, e.g., calcium (3). The converse of this also appears to be true, and agents which improve the solubility of a radicle in the intestine also improve its absorption (4). This all sounds so obvious when it is enunciated that it is essential to think oneself back into the state of knowledge existing only a few years ago to appreciate the work undertaken to establish it. The whole story has not yet been told, for biological systems are so complex that in special circumstances general principles may appear to be over-weighted by local conditions. Work along these lines is still being industriously carried out (5, 6).

Calcium and Magnesium

The absorption of calcium continues to occupy a great deal of attention. This is very appropriate, for the world is facing a shortage of milk, and the quantity and availability of the calcium in all other foods has assumed greater importance. It has been shown, for example (7), that up to 200 mg. of calcium a day may be obtained from drinking water in districts where the water is hard. This is as much as there was in the adult milk ration in Britain during the winter months of 1942 and 1943. Between one hundred and two hundred Indian vegetables and fish have been analysed for calcium and phosphorus during the past year (8, 9, 10). It seems to be agreed that rats can utilise the calcium of vegetables (so long as they do not contain oxalic acid) between 70 and 90 per cent as efficiently as the calcium in dried skimmed milk (11, 12). Some work on humans has been carried out (13 to 16). So far as they go, the experiments of Basu *et al.* (13, 14, 15) indicate that the ingestion of the bones of small fish, and of lime chewed with betel nuts, has a beneficial effect on calcium absorption. Breiter *et al.* (16) concluded that the calcium in carrots was about half as well utilised by adults as the calcium in milk.

The necessity for making the available wheat feed as many mouths as possible has prompted a number of investigations. McCance & Widdowson (17) carried out prolonged studies with humans to investigate the absorption of calcium from diets containing large amounts of flour of 92 per cent extraction. Both calcium and magnesium were absorbed less freely from these diets than they were from similar ones containing white flour. Flour of 85 per cent extraction has also been shown to have an inhibitory action upon the absorption of calcium (18). Considerable decalcification of the bones of the general population has recently been reported from South Africa (19), and attributed to the increased consumption of long extraction flours. An increase in the stigmata of calcium deficiency was predicted if countries should be forced to adopt a whole wheat flour (17) and Jessop and others (19 a) have now shown that such an increase has in fact taken place in Eire. By adding sodium phytate to white flour, and by removing phytic acid enzymatically from brown flour, it has been shown (17, 20) that the deleterious effect of brown bread on the absorption of calcium and magnesium in man is entirely due to phytic acid. The insolubility of the calcium salt of this complex acid radicle enables it to immobilize calcium in the intestine from the rest

of the diet and so prevent its absorption. Similar results have subsequently been obtained by Wang *et al.* (21). The effect could be completely overcome by adding calcium carbonate to the flour, and this is now a national practice in Britain. In recent months a preliminary note has appeared (22) stating that the phytic acid in oatmeal is less deleterious than that in wheat. This is very surprising, especially in the light of the well-known rachitogenic effects of oatmeal (23), and it would be well to reserve judgment until the full text of the experiments has appeared.

Working entirely with normal human adults, McCance & Widdowson (24) have shown that, whether the subjects were or were not "in balance," a rise or fall in the absorption of calcium or magnesium was always accompanied by a change in the same direction in the amounts of these metals excreted in the urine. Apart from its general significance, this observation should be of technical value to those interested in absorption, for the demarcation of faeces, and consequently the measurement of absorption, is notoriously difficult.

Kosman & Freeman (25), using a chronic colon fistula in dogs, obtained some evidence that calcium was actively excreted by this part of the intestine when it was irritated with mustard oil. If this is true, it must be due to the irritation set up by the mustard oil, for Nicolaysen (26) had previously shown that normal dogs excrete negligible amounts of calcium into the large intestine.

Following up an observation that certain amino acids increased the solubility of calcium and magnesium salts (27, 28), McCance, Widdowson & Lehmann (4) have shown that the proteins in the diet facilitate the absorption of both these metallic ions. This fact offers an explanation of a number of earlier observations and is probably the clue to some of the more recent results of Boyle & Wesson (29). These authors found that vitamin D improved the retention of calcium by rats on a high carbohydrate diet, but not by animals on a high protein diet. The fact that vitamin D improved the structure of the bones and teeth of all the animals is evidence that it must have an action on the bones as well as on the intestinal epithelium. According to Sobel *et al.* (30), vitamin D influences the total amount of calcium deposited in the bones, but the relative proportion of carbonate and phosphate in them depends upon the dietary Ca/P ratio. Incidentally, Patwardhan & Chitre (31) have recently disputed that the absorption of calcium is improved by vitamin D, but the evidence in favour of this action of the vitamin would appear to be overwhelming.

A paper which may help to clear up some confusion and to standardise technique (32) has shown that when rats of either sex are short of vitamin D, the femur, with its relatively large metaphysis, is calcified more poorly than the humerus, but when the deficiency is one of calcium or phosphorus, but not vitamin D, both bones are equally calcified.

Enormous doses of vitamin D continue to be given both to dogs and humans (33, 34), but the main fact which is emphasised by such work is the tolerance of adult animals to this form of abuse. Quite small doses appear to be sufficient to produce hypercalcaemia in rats if the diets contain large amounts of calcium (35). On the other hand, Ritchie (36), as part of a large analytical investigation of human milk, showed that administering calcium did not raise the concentration of this element in the milk unless vitamin D was given concurrently. The milk of mothers whose infants had rickets contained normal quantities of calcium and phosphorus.

Bunkfeldt & Steenbock (37) have taken up once more the question of the antirachitic effects of vitamin D–free fats, and they have found that these fats may be harmful or beneficial according to the Ca/P ratio in the diet. They obtained worse and worse calcification as the Ca/P ratio was raised above 1, and beneficial effects with lower ratios. Their results are not quite in line with those of Booth *et al.* (38), but Bunkfeldt & Steenbock do not consider there is any essential contradiction. There is something rather interesting in these effects. One would like to explain the decreased calcification at high Ca/P ratios as being due to the fatty acids precipitating calcium in the gut, but the improved absorption is more difficult to explain. Calcium may, however, form soluble complexes with fatty acids, just as it has recently been shown to do with amino acids (27, 28). With these results must be considered those of Jones (39) and of French (40, 41). The former found that fats improved the absorption of calcium, but the latter that calcium absorption deteriorated as the amount of fat in the rats' diets was stepped up.

Nicolaysen (42) has published some work which confirms the suggestion (43) that the amount of calcium absorbed by young rats depends upon the degree to which the tissues were previously saturated with the element. This effect was not observed in adult rats, even when they were pregnant, and was only detected in young animals when they were given vitamin D. As one might have expected, such animals utilised calcium lactate better than the stearate, although

animals saturated with calcium did not appear to do so (44). Furthermore, the same author has found (45) that in the absence of vitamin D the citric acid content of bone falls to a low level. In his opinion these changes in citric acid content do not depend upon calcification, so they must be due to the influence of vitamin D upon the metabolism of the bone stroma.

Seasonal changes in calcium absorption have been detected in some individuals (46). In these people, absorption was always better in July than in February. Magnesium was absorbed equally well all the year round. So far so good, but 2000 I.U. of vitamin D per day (as calciferol) did not make the winter absorptions any better, and the authors were forced to conclude that these changes must have been due to some variation in these persons' resistance to the vitamin D already inside them. Seasonal changes in calcium metabolism are of course well known in birds, and have recently been studied once again (47).

Braude *et al.* (48, 49) have discovered a new rachitogenic agent in yeast. They were studying the ability of yeast to supply the whole of the pig's requirements for dietary protein, and in doing so they included diets containing up to 20 per cent of dried yeast. Animals on such diets invariably developed a low-calcium form of rickets. They were partially protected by calcium carbonate, and completely by cod liver oil. The authors are satisfied that the effects were not due to a surfeit of phosphorus in the diet, but the nature of the agent which prevented the absorption of calcium is still quite obscure. It can hardly be the yeast protein. Bovine rickets is a very rare disease in England, but some cases have been recorded in shorthorn heifers, pail-fed on a proprietary food of unknown composition (50).

In view of the many factors which are now known to influence calcium absorption, the approach to the problem of calcium requirements by means of balance experiments becomes more and more unsatisfactory. If the intakes of phytic and oxalic acids, and of protein and fat, for example, are not defined, the determination of the calcium requirement has little scientific meaning and, if they are defined, the data have little practical importance, for the diet becomes such an artificial one. When, moreover, one considers the enormous differences which may exist between the ability of any two people to absorb calcium (17, 20), the true magnitude of the problem begins to appear. Nevertheless, the subject is one which continues to occupy the attention of a special group of investigators, and the calcium bal-

ances of premature infants (51) and of young college women (52) have received attention within the period under review. Aspects of calcium requirements have been reviewed by Robertson (53) and by Duckworth & Warnock (54). The latter authors have also considered the question of magnesium requirements, and they concluded that a great many people in the British Isles were getting less magnesium than they needed before the war. This seems to be going a little too far but, be that as it may, children's diets in Madrid in 1941 were undoubtedly grossly deficient in calcium (55), and the same is true of many parts of India (56).

The influence of the well-being and cellular activity of the whole animal on the amount of calcium absorbed is emphasised by some work of Krishnan (57), who found that intraperitoneal injections of two to five units of antuitrin growth hormone (Parke Davis & Co.) into rats and guinea-pigs increased their absorption and retention of calcium as measured by balance experiments. It is, however, difficult to evaluate this work, for the growth and weight changes induced by the hormone are not given. The influence of estrogens and androgens on the skeletal system has been reviewed (58).

The relation between calcium metabolism and vitamins other than D has received some attention. The bony changes induced by vitamin A deficiency have been further explored in young dogs (59). It is evident that this vitamin exercises an important controlling influence upon the growing bone of this species. In its absence the growth of the bones of the skull becomes exaggerated and incoordinated. The foramina for the cranial nerves are always involved and, in consequence, the nerves become stretched, twisted, and compressed. In this process the sensory suffer more than the motor nerves. In spite of this bony distortion, calcification may be quite normal if enough calcium, phosphorus, and vitamin D are supplied. Vitamin C also seems to be involved in calcium metabolism (60 to 64). Reid (65) has reviewed the work on this subject. Bourne (61), working particularly on the formation of new bone, uses the term "positive" balance to describe what is to most people a "negative" balance, and this makes part of his article a little confusing.

Calcium deficiency has been shown to produce small multiple lesions in the antrum of the rat's stomach (66), and its effect on pregnancy and lactation in the rat has been studied (67) without any very unexpected findings. Deakins & Looby (68) did not find that pregnancy led to a decalcification of human dentine, but their subjects were

not living under very stringent conditions; had they been so positive results might have been obtained, and lactation might have been a more searching test. It can be shown to be so in the rat (67). In adult rats prolonged calcium deprivation makes the molar teeth friable and loose, but not decalcified, and the incisor teeth remain normal. The alveolar bone becomes decalcified (69).

A paper has appeared on the calcification of the human placenta (70) and two on the qualitative composition of urinary calculi (71, 72). Inclan (73) has described three cases of calcinosis, and has reported finding large aggregations of calcareous material and fluid in cyst-like cavities. Such cases have been reported before (74), and Inclan's statement that the fluid contained calcium carbonate in suspension must surely be a mistake: calcium phosphate is much more probable (75, 76). The author seems to have missed a chance of extending observations which were made many years ago in England (76). More recent studies by Watchorn & McCance (unpublished) on a case which was of a similar type, but less satisfactory for purposes of investigation, have tended to confirm their original findings. Liu & Chu (3) have studied the calcium metabolism of five patients with renal rickets, and Follis & Jackson (77) the osseous rarefaction secondary to nephritis in adults. Two more cases of the fibrous dysplasia of bone associated with endocrine and cutaneous changes (Albright's disease) have been fully described (78). Its etiology is still very obscure, but some fresh manifestations have been brought to light. There must be a wealth of biochemistry behind the widespread changes which characterise this disease. Sanguinetti (79) has given a review of some of the clinical and nutritional aspects of mineral metabolism, and he has devoted a special section to osteoporosis.

The ultrafilterable magnesium in human serum has been studied by Dine & Lavietes (80) and also by Cope & Wolff (81). The former pair have claimed to confirm earlier work that in the hyperthyroid state an excessive amount of magnesium is bound, and that in myxoedemà practically all the magnesium is ultrafilterable. The latter pair are in complete disagreement, and at present there are no signs of a reconciliation.

Magnesium appears to be able to displace calcium from myosin, and by so doing to inhibit the enzymatic breakdown of adenosine triphosphate (82). This has a practical value for those wishing to prepare adenosinetriphosphate, and a theoretical one for those interested

in muscular metabolism or magnesium anaesthesia (83). This work may explain why 5 to 10 cc. of magnesium sulphate given intravenously have been found to be of therapeutic value in paroxysmal tachycardia (84).

The variations in serum magnesium in health and disease have been reviewed by Haury (85). A rather unusual piece of analysis has been carried out by Williamson & Gulick (86). They separated the nuclei from the cells of the thymus gland by mechanical and physical methods and analysed them for calcium and magnesium. The dried nuclei contained about 1.35 per cent of calcium and 0.09 per cent of magnesium.

Iron

The absorption of iron, like that of calcium, is liable to be interfered with by the precipitation of the metal in the intestine. In this respect the phosphates and phytates are of practical importance. Moore *et al.* (87) had previously shown that, in contrast to the effects of soluble salts, large doses of phosphates of iron did not raise the level of iron in the serum. It has recently been demonstrated by means of human balance experiments (88) and serum iron curves (89) that phytic acid will depress the absorption of iron. Rats have also been studied in this connection (90, 91), but it is frequently forgotten that the results are not applicable to man, for the rat secretes a phytase into the intestinal tract. Phytase, however, does not seem to be able to break down phytates when these are present in an insoluble form (92), and this probably explains why ferric phytate is not so well utilised by rats as other iron salts. Nakamura & Mitchell (91) found that the pyrophosphate of iron was as well utilised as the chloride, but Street (93) has been unable to confirm this. Freeman & Ivy (94) observed that antacids such as calcium carbonate and aluminium hydroxide reduced iron retention in anaemic rats, but that aluminium phosphate did not and magnesium trisilicate only did so to a very slight extent. One would expect a reduction in the acidity of the stomach to lead to the precipitation of ferric hydroxide and other iron compounds, but it is difficult to see why the various antacids should have such different effects.

It now seems generally agreed that the body has no physiological method of regulating the excretion of iron, and that, in point of fact, very little ever is excreted (95). During the past year the evidence

has been strengthened by a study of iron balances in rabbits after subcutaneous administration of iron triethanolamine chelidamate (96), by following the alimentary exchanges of a patient who was being given large and frequent transfusions (97), and by analysing the livers and spleens of persons who had had secondary anaemia. Not only were these patients not short of iron, but there was actually a large accumulation of the metal in their storage organs (98, 99). The amount in the body must, therefore, be regulated by the amount absorbed, and Hahn *et al.* (100) have published an account of an interesting, although still very incomplete, attempt to solve this problem by means of radio-active iron. They have found that the amount of iron absorbed by dogs depends upon the degree to which the mucosa has previously been saturated with the metal. Others (42, 43), it will be recalled, have made a similar suggestion about calcium. Saturation by iron is rapid, and a large dose given a few hours in advance may block the absorption of a second dose containing the radio element. The removal of blood does not at once "desaturate" the mucosa, but this follows gradually as the iron stores are depleted to make good the loss of blood. Hahn *et al.* found that iron was rapidly absorbed from a pouch of the stomach, which is rather a novel conception of the function of this organ, and they followed the changes in the serum iron induced by administering the metal in this way. They detected that the peak of the serum iron curve might be reached and passed before the iron was removed from the pouch, and they hailed this as further proof that "saturation" of the mucosa will prevent further absorption. This evidence does not seem wholly satisfactory for, after all, the peak of a blood sugar curve is reached long before the glucose is all absorbed from the gut. Storage of iron may not be an altogether passive process, and it must have some part to play in the regulation of serum iron.

The "balance" technique still continues to be used for the study of iron requirements (52, 101), but, since iron does not seem to be excreted once it has been absorbed, it is hard to see what conclusions can be drawn from such experiments. Johnston & Roberts (102) have appreciated the difficulty, and have abandoned this method altogether, which seems the only logical thing to do. They used the haemoglobin levels as their index of the subjects' iron sufficiency, but there are fallacies about this method also, and at its best it is an indirect one. It is, however, convenient for large scale investiga-

tions (103). The value of food tables for calculating iron intakes has been studied (7, 104). It has been shown (7) that the figures so obtained must often be lower than the actual intakes because of contamination from cooking utensils.

Fowler & Barer (105, 106) obtained some rather unexpected results when they followed the rate of haemoglobin regeneration in blood donors who were being treated with iron. They found, in confirmation of previous workers, that when blood was first withdrawn the administration of iron shortened the period required for complete regeneration, but they also found that this beneficial effect was not so obvious after the second removal of blood, and negligible after later ones. The puzzling feature of these observations is that the donors who were treated must have been absorbing far more iron than the controls, and many times more iron than they can have required for the haemoglobin synthesis. Their stores of iron were full and their serum irons were high. Why had the metal lost its charm? The serum iron in pernicious anaemia has again been shown to be high and to fall with treatment, sometimes to very low levels (107). The hypochromic anaemias, so often associated clinically with vitamin B_1 and B_2 deficiencies, have been found to respond to iron, and not to respond any better when yeast had been given at the same time (108). Unlike some other animals, man does not seem to carry a reserve of red cells in his spleen (109).

Two authentic cases of familial idiopathic methaemoglobinaemia have been recorded in Ireland (110), and it has been discovered that the abnormality may be "cured" by the continuous administration of ascorbic acid. Further investigation of these cases may throw light upon the metabolism and the oxidation-reduction mechanisms in the normal red blood cell. Haemin has been added to the list of substances which augment the action of the gonadotropic hormone of the pituitary (111).

Two new iron compounds have been isolated during the past year. One, a protein, which crystallises readily as the cadmium salt, was first prepared from the spleen of the horse (112). It has now been obtained from a number of organs and from several species, including the liver of man (113, 114). The protein has been obtained free of iron (115), and its molecular weight is about 500,000. It is considered that the iron is present as colloidal ferric hydroxide interspaced in the crystal lattice of the protein (116, 117). The other iron compound has been isolated from red human hair (118). It is a purplish

red pigment, thought at present to be a phenolic derivative. The details of its structure will be received with interest.

Manganese and Cobalt

The use of radio-active elements has extended our knowledge in several directions (119). The absorption and excretion of manganese and cobalt by rats has been investigated in this way (120), and by balance experiments in man (121). Both metals are absorbed somewhat incompletely when they are taken by mouth. It seems clear that once cobalt has been absorbed it is excreted mainly by the kidney. Small amounts, however, also find their way into the bile and juices of the intestine. Manganese is predominantly excreted by the liver into the bile; minor amounts enter the intestine with other glandular secretions, and still smaller quantities are eliminated by the kidney. After equivalent dosage some human beings appear to excrete manganese less rapidly and less completely than the rat.

Shils & McCollum have reviewed the subject of trace elements (122) and made a study of the effects of manganese deficiencies in the rat and mouse (123). This work was undertaken to try to find out why the results of other investigators had been so contradictory. In this they were not altogether successful, but they came to the conclusion that rats and mice behaved alike, and that the discordant findings were due to differences in the intake of manganese rather than in the species of animal. Some of their animals became incoordinated and lost their sense of equilibrium, a sign of manganese deficiency hitherto undescribed. Boyer *et al.* (124) found that manganese deficiency produced stunted growth and sterility, but that it did not reduce the activity of intestinal peptidases. The deficient rats had less arginase in their livers, as determined after considerable dilution in *brei.* This does not prove that the arginase would have been less active *in vivo,* for the deficiency may only have been unmasked, so to speak, by the dilution. Other work has gone to show that the activity of the bone phosphatase in chicks is linked up with manganese catalysis, and that the over-activity of this enzyme, so characteristic a feature of rickets, only shows itself in the presence of enough manganese (125). Rudra (126) holds the opinion that the guinea-pig's intestine synthesises ascorbic acid, and that manganese is essential for this. He has carried out some experiments on a few animals and claims to have proved his theory. Manganese

has been shown to produce hypoplasia of the dental enamel of the rat's incisor. The effects, unlike those of strontium, are limited to the apical fourth of the zone of matrix formation (127). The amount of manganese in cow's milk can be doubled by adding 30 to 60 gm. of manganous sulphate to the daily food of the animals (128).

Polycythaemia induced by cobalt has been shown to improve the working performance of anoxic rats, but the performance of the animals was at its best after the cobalt treatment was discontinued and before the polycythaemia subsided (129). There has been some development in our knowledge of "Coast" disease in sheep. It seems that the cobalt is not curative when given by injection, only when given by mouth. Thus the element probably acts upon some of the organisms in the rumen, not on the host (130, 131), and this explains perhaps why horses do not suffer from this disease.

Zinc

Zinc is one of the metals which is undoubtedly excreted by the intestinal tract. McCance & Widdowson (132) found that the amounts in human urine only became significant when much albumin was being excreted. Sheline, Montgomery, and others (133, 134, 135) have now made some considerable advances in this field by injecting radio-active zinc into dogs and mice. They have shown that injected zinc accumulates in the mucosa of the intestine and is concentrated by the pancreas from the plasma to the pancreatic juice. It also accumulates in the liver, but is not excreted in the bile to any extent. These studies of the movements of zinc about the body reveal what can be done with radio-active elements, but they also show how little we understand at present of the physiology of this metal, for, as the authors themselves point out, it is quite impossible at the moment to explain the distribution and movements of the injected radio-zinc in terms of the compounds of the body such as insulin and carbonic anhydrase, which are known to contain the metal.

Lead

Another example of the way in which one radicle will precipitate another and so prevent its absorption is found in the case of lead. Pectin, which has an insoluble lead salt, has been shown to depress the absorption of this metal (136). An experimental study of lead poisoning, and of the primary and secondary distribution of

ingested lead throughout the body, has been made by Fairhall & Miller (137, 138). Sodium citrate has been held to be of help in the treatment of industrial lead poisoning in man (139, 140). Following its administration the level of lead in the serum fell, and there was an immediate amelioration of symptoms. In spite of the fact that most of the classical signs were not present, lead poisoning has been attributed to the absorption of the metal from a bullet lodged in the sphenoidal sinus eight years before (141). An outbreak of lead poisoning in cattle has been described due to contaminated silage. The milk of a survivor contained 2.26 p.p.m. (142).

Human hair has been analysed for a number of "trace" elements (143, 144), and black hair has been found to contain more lead than fair or grey hair.

Copper

Sachs *et al.* (145) have reviewed the subject of copper in human blood, and have shown that the amount varies inversely with the iron at all ages and in all pathological conditions so far studied. Both metals of course accumulate together in the liver in haemochromatosis (146). Gross *et al.* (99) have reported that tumour tissue is high in copper. The metal has been shown to hasten the segmentation of *Arbacia* eggs (147), and work has gone on upon the way in which copper salts promote ovulation and pseudopregnancy in the rat and rabbit (148). This action can only be demonstrated if the animals have been under the influence of their own or injected estrogens, and the site of the action is thought to be the nervous system.

Molybdenum

The studies of the "teart" pastures of Somerset, England, have given us a fascinating chapter in animal pathology and a very rapid therapeutic success (131, 149 to 153). Cows kept on these pastures develop intractable scouring, lose weight, and go down-hill generally until they die. The cause has been found to be poisonous quantities of molybdenum in the soil and herbage. The conditions which promote or restrict the uptake of molybdenum have been defined, but the most satisfactory feature of the whole investigation is the discovery that 2 gm. of copper sulphate by mouth every day will protect the animals, and themselves give rise to no ill effects. No account has yet been published of the effects of copper given by injection.

Fluorine

Most of the work on fluorine has centred round its capacity to induce mottled enamel and, in optimum doses, to prevent caries without disfigurement (154 to 158). It seems an important point that the halogen can protect the teeth even after they are fully calcified (131, 159). Irving (160) has briefly described some effects of fluorine on the teeth of rachitic rats, which has introduced a new technique, and probably new problems. McClendon & Foster (161), working on themselves, showed that fluorine is stored in the bones as well as the teeth, and Linsman & McMurray (162) have described the first case in America of skeletal involvement in chronic fluorosis. Cases had already been reported in England by Kemp *et al.* (163). The importance of a good diet, if skeletal deformities are to be avoided when fluorine-containing waters are being drunk, has been emphasised (164). Mottled nails, as a sign of fluorosis, have been described by Spira (165). The water supplies of a number of towns in England and Wales have been analysed for fluorine (166), and it has been claimed that fluorides can be removed from water by boiling (167). This finding should be treated with reserve until it has been confirmed (168).

An outbreak of acute fluorine poisoning, which affected 263 persons and caused 47 deaths, has been reported (169). An insecticide was mistaken for dried milk and incorporated into scrambled eggs. This is not the first accident of its kind in the United States, and it looks as though some further legislation might have to be introduced to protect the public from such carelessness or stupidity in food processing and handling.

Iodine

Some very interesting discoveries about the metabolism of iodine have been made in the last 18 months, one of which may have most important therapeutic applications. This was the result of an investigation by Astwood (170), who showed that thiourea and thiouracil both inhibited the production of thyroxin to such an extent that patients with thyrotoxicosis could rapidly be brought into a state of metabolic normality. One of three patients developed agranulocytosis, but recovered. The work has been confirmed by Himsworth (171), and will probably have been reconfirmed again and again by

the time this article appears in print. Further work and experiences with these compounds are awaited with interest, and also more detailed reports of the therapeutic possibilities of radio-active iodine (172). Another new and much more physiological compound with anti-thyroid activity has been discovered by Carter *et al.* (173). This substance is paraxanthine, and there is one very unusual feature about its activity which may ultimately turn out to be of great significance. It will only antagonise thyroxine when the two substances are present together in definite proportions. This makes it difficult to apply clinically, for the "active" dose has very narrow limits. Sharpless & Anthony (174) have failed to confirm a claim that chlorides were goitrogenic.

Two groups of workers have studied the fractionation of the iodine compounds in serum. Eighty-five per cent (175) or more (176) of the serum iodine is bound up with the proteins, and the normal range is 5 to 12 μg. per 100 cc. In normal persons treatment with Lugol's solution raised the soluble iodine in the serum without altering the fraction bound to protein. Persons with thyrotoxicosis have more than the normal amount of iodine in their serum, and practically all of it is "bound." Lugol treatment reduced the precipitable iodine in the serum of hyperthyroid patients, but only if they were making a good clinical response.

Working with radio-iodine, Morton & Chaikoff (177) have shown that tissue slices of the thyroid gland of the sheep, dog, and rat rapidly synthesise thyroxine and diiodotyrosine, and others (178) have demonstrated that this synthesis is inhibited by the sulphonilamides. It has also been shown that synthesis of both compounds must not only go on in the thyroid (179) but also in other organs (180), which seems to be rather a fundamental contribution to developmental and comparative physiology. Leblond *et al.* (181) have found that adult rats can be induced to synthesise thyroxine much more rapidly by keeping them at 0°C. for a few days. This argues for greater thyroid activity and explains why persons in cold climates demand more calories. It appears that iodides are selectively concentrated and excreted by the salivary glands of man (182, 183). It is difficult to see any biological value in this performance, for the iodide will only be reabsorbed lower down the alimentary canal, but some such mechanism may explain the well-known presence of thiocyanates in salivary juice. These glands will not excrete the iodine in tetraiodophenolphthalein, although of course the liver selectively does so.

Other Trace Elements

A hundred different foodstuffs eaten in India have been analysed for arsenic (184). Sea fish tend to be the richest and vegetables the poorest sources. The rate of excretion of gold salts has again been shown to depend upon their solubility (185, 186). Tin salts in toxic doses have been given to rabbits (187). It has been confirmed that strontium behaves like calcium as regards absorption and excretion (188), and this element has been used to mark the rate of growth of dentine (189). Radio-strontium has been claimed to have therapeutic possibilities in the treatment of secondary growths in bone (190, 191) and phosphates have also been tried (192), but it is much too early to appraise this form of therapy. Youmans & Delves (193) have found that barium chloride and nitrite, but not other salts tested by them, tend to make *Staph. aureus* produce small colony variants. The anticoagulant actions of neodymium, lanthanum, and cerium have been investigated in man, but their use for such a purpose is contraindicated because of their toxicity (194). The effects of sodium sulphide on the metabolism of tissue slices has been studied by Smythe (195), and sodium nitrite in the doses required for food preservation has been shown to do rats and cats no harm (196).

The Cell and Its Environment

A survey of the alkali metals necessarily involves a simultaneous review of chlorides, bicarbonates, and water, and this in turn demands some consideration of the constitution of the fluid compartments of the body, and of the way in which they are regulated by the kidney.

Extracellular fluid volume.—Two groups of workers have studied the determination of the extracellular fluid volume (197, 198). The thiocyanate method can be standardised, but, whatever the state of hydration of the animal, it always gives results higher than those given by radio-active sodium, and these again are above the figures obtained by the use of radio-active chloride. Without further investigation no method can be considered to give absolute results, but all may have their uses for comparative purposes. Even so, all methods should be considered in the light of what is now being discovered about the factors which alter the permeability of the cell membrane and the distribution of ions within the body (199 to 203).

By a study of all the ions, Childs & Eichelberger (204) set out to discover whether normal pregnancy in dogs produced any tendency

to edema formation. They came to the conclusion that 15 per cent of the muscle mass was extracellular, whether the animals were pregnant or not, and that all reacted to the administration of large amounts of isotonic saline in the same way. The exposure of monkeys and rats to low temperatures has been observed to produce some rather complex exchanges of water between the cellular and extracellular compartments of the body (205). In the early stages the serum proteins and chlorides rose and water passed into the cells. These changes were at their maximum when the body temperature was about 30°C. At lower temperatures, when the hypothalamus ceased to respond to reflexes, water passed out of the cells until the serum chlorides fell to less than their normal levels.

The internal environment.—Several groups of investigators have changed the osmotic pressure and the pH of man's internal environment and studied the effects. Kirsner and his co-workers (206) have induced both salt deficiency and alkalosis by the continuous aspiration of gastric juice from patients with peptic ulcers. By these means the serum sodium was reduced to 115 m.eq. per litre in one case, and the serum chloride to less than 60 m.eq. per litre. The other changes were on the whole similar to those described by previous investigators. The patients lost a considerable amount of weight and felt listless; their plasma proteins and haematocrit values rose, as did also the bicarbonate and the pH of the serum. In spite of these last two changes the urine of one patient was acid throughout, which conforms with clinical experience, and with earlier experimental work (207). It is evident that during a period of salt deficiency the kidney will not excrete sodium to correct a simultaneous alkalosis. The regulation of the body's osmotic pressure comes first. Kirsner *et al.* make a great deal of the fact that the blood urea of these patients did not increase, and they attribute this to the fact that the salt deficiency was induced slowly, over a period of twelve days. While this may have been a factor, it is certainly not the only one. Some of the subjects of McCance & Widdowson (208) were made salt deficient over a period of eight days or more, and they showed signs of renal deterioration only towards the end. Kirsner's subjects had lowered blood pressures and all the signs of a diminished blood volume. The stage was therefore set for a rise in blood urea and a fall in urea clearance and, if the authors did not detect it, individual idiosyncrasies were probably responsible. As a matter of fact both the patients who were extensively studied showed a rise of blood urea over their normal levels,

and probably a fall in clearance at the same time, but the authors chose to minimise it.

Kirsner's other papers have shown that the administration of calcium carbonate and aluminium hydroxide does not throw enough strain on the mechanisms of acid base regulation to produce clinical alkalosis (1), and that the administration of sodium chloride will prevent the onset of alkalosis during the treatment of peptic ulcers by the aspiration of gastric juice (209). This might have been anticipated from the clinical and experimental work of many previous investigators (207). Salt deficiency in dogs has been reported to make for subnormal oxygen consumption by interfering with the rate of dissociation of oxygen and haemoglobin (210, 211). Sanguinetti (79) has written enthusiastically about the beneficial effects of low mineral diets in cases of heart failure and nephritis.

A good deal of work has been carried out on both sides of the Atlantic on the effects of dehydration (212). Most of this has been inspired by the exigencies of the war, and some of it has not yet been released for publication. A study has been made of the effects of water deprivation by Ladell (213). Subjects were maintained on diets similar to those likely to be available in lifeboats. They contained insufficient calories and very little salt. The experiments were planned to determine whether it was beneficial in these circumstances to drink small quantities of sea water in addition to the limited ration of fresh water. Ladell found that the volume of urine did not fall after the first day. Drinking up to 400 cc. of sea water (12 to 14 gm. of sodium chloride) increased the volume of urine, but not by more, and often by less, than the volume of sea water taken. All the extra sodium chloride was excreted. The increase in the amount of water excreted improved the urea clearances, and the extra water to drink, even though it was highly salted, had a good psychological effect. Black, McCance & Young (214) have also studied the physiological effects of a water shortage. They have confirmed the experiments of Nadal *et al.* (215) that during three or four days of water deprivation the body may lose 10 per cent of its weight without any appreciable reduction in the blood volume, and they found no significant changes in blood pressure or glomerular filtration rate (216). McCance & Young (217) tried the effect of varying the salt intake during water deprivation, and found that increasing the intake always led to a rise in the volume of urine. The extra water came from the body fluids. The authors also studied the effects of rehydration in

stages. When subjects, who had been deprived of water for some days on a diet containing 5 to 15 gm. of salt, were given somewhat less water than the amount of weight they had lost, their urine volumes fell. The amount passed in the first 24 hours of rehydration was always smaller than it had been during the days of dehydration. The accompanying changes were a large fall in the percentage of chlorides in the urine and a rise in the percentage of urea. The latter is particularly interesting because it was associated with a fall in the serum urea. The authors considered that the reduced output of water and the increased percentage of urea in the urine were both due to a fall in the excretion of sodium chloride. Such a fall would certainly make for a diminution in the volume of the urine for reasons which have just been given. So much less salt presented itself for excretion, however, that the percentage of chlorides in the urine fell. This allowed the kidneys to raise the percentage of urea. The authors are not in agreement with Ladell's conclusions about the beneficial effects of sea water. The supposedly beneficial effects can only be observed when the diet contains very little salt and the intake of sea water is limited. It is hard to see how the administration of a strongly hypertonic solution can possibly benefit a man who is already short of water. The extra salt may be excreted, but only at the expense of a further rise in the osmotic pressure within the body, and this cannot be beneficial. Large amounts of sea water are admitted by all to be strongly contra-indicated.

Interesting but complicated effects of sodium chloride upon the sugar metabolism of rats have been reported (218). Sugar was given intraperitoneally in water or in saline and the effects compared. The salt decreased the blood sugar and increased the deposition of glycogen. It also decreased the amount of sugar which was transformed into unidentified products. One wonders how much of these effects of sodium chloride was due to the fact that it corrected the salt deficiency caused by the intraperitoneal glucose (219). Primary atypical (or virus) pneumonia, alias acute pneumonitis, has been shown not to provoke a disturbance of chloride metabolism as acute pneumococcal pneumonia is well known to do (220).

The excretion of chlorides and water by the kidney has been studied in dogs (221), normal and hypertensive subjects (222), and in students during and after exercise (223). The last study embraced phosphates, ammonia, creatinine, and other urinary constituents. The chloride excretion has again been invoked as a help in

the differential diagnosis of Addison's disease, and it is clear that the conditions originally laid down were not sufficiently stringent (224). The inability of the infant's kidney to maintain a normal internal environment under conditions which would not be considered unfavourable in adult life (225) has again been noted. It has become clear (226) that the urea and mineral clearances tend to be very much below the adult levels at birth, and that the infant cannot produce a concentrated urine, even when short of water. These are the reasons why diarrhoea and vomiting and injudicious saline therapy (227) may produce such remarkable changes in the composition of the serum.

The blood picture and the internal environment of the carp and trout have been subjected to a detailed study (228). The isolated crop of the insect, *Periplaneta Americana,* will only survive and remain active if it be immersed in a solution containing both potassium and calcium (229). There is nothing surprising in this, but it is evident that this insect requires calcium even if others do not (230).

Mineral exchanges of unicellular organisms.—*E. Coli* (231), like yeast (232), has been shown to take up potassium in large amounts during the stage of glycogen synthesis at the commencement of fermentation, and to release it again into the medium as the fermentation proceeds (233). A very pretty explanation of these and similar observations on muscle has been advanced (234).

The unicellular alga, *Chlorella pyrenoidosa,* has been shown not to require calcium for its growth and reproduction, and to be able to replace some of its potassium by sodium when the supplies of the former are inadequate (235). The effects of the halide environment on *Penicillium sclerotiorium,* and upon the production of its chlorinated metabolic product have been studied (236). The organism will not substitute bromine or iodine for chlorine in the organic product.

The washed cells of *B. cereus* rapidly die out in distilled water, but they are protected for some time by solutions of sodium chloride as dilute as 0.00001 *M* and up to 0.3 *M* (237). Observations of this sort recall those of others (238, 239), on much more complicated animals such as goldfish.

Potassium

A considerable amount of work has been carried out on the potassium ion (240). It has been shown to have a catalytic action in the

transference of phosphates from 2-phosphopyruvate to the adenylic system (241). Such work demonstrates, if such a demonstration were needed, that potassium is not retained within the cell merely to regulate the osmotic pressure. Experiments on a few mice have shown it to be very difficult to raise the body potassium by dietary means (242).

After the release of a tourniquet which has been applied for orthopaedic operations both the serum potassium and the inorganic phosphorus have been shown to rise (243). Prolonged application of a tourniquet to a dog's limb leads to similar but greater changes in both radicles (244, 245). The potassium may reach 8 m. *M* per litre, but this is not enough to cause death, for in cats, poisoning, as indicated by the electrocardiogram, only begins to show itself when the level in the serum is raised slowly by injection to 11 m. eq. per litre (201). Trauma may also cause a large increase in the serum potassium, and if an animal is on the point of death from secondary shock, very high figures for this ion may be obtained. It is not, however, thought that this change is the actual cause of death. It is the old story of *post hoc* rather than *propter hoc* (244, 246). Lyman (247) found that radioactive potassium penetrated abnormally rapidly into a mammalian muscle which had been denervated for some days. He associated this with the ease with which potassium contractures can be induced in such muscles. Of rather more pharmacological interest is the discovery that, if the heart of the dogfish, which is not supplied by sympathetic fibres, has been brought to a standstill by an excess of potassium ions, it can be started up again by epinephrine (248). In "winter" turtles (249) the heart has been shown to be relatively insensitive to lack of potassium and to be hypersensitive to vagal stimulation. In the summer the turtle's heart stopped beating soon after potassium was withdrawn and was not so responsive to vagal stimulation. Only in winter could the heart be made to stop in systole by the administration of an excess of potassium, together with one of the cardiac glucosides.

Potassium deficiencies have been studied by several groups (250 to 254). Rats deficient in this metal developed histological lesions in the heart which, oddly enough, were prevented by a thiamin deficiency but were made worse by exercise. After the exercise violent contractions of the skeletal muscles were sometimes noted. Rubidium prevented the development of these histological lesions in the heart, but the doses given made the animals very irritable; in ten days they

began to have fits and died less than a week later. The cardiac lesions of desoxycorticosterone acetate (D.O.C.A.) poisoning are probably in essence those of potassium deficiency, for they can be prevented by adding potassium chloride to the drinking water (202). A potassium deficiency leads to renal hypertrophy (251) and prolongs the life of nephrectomised animals (250). This work should be read in conjunction with that of Keith *et al.* (255). Of interest in this connection is the work of Talbot *et al.* (200), who set out to study the action of androgens upon a girl with Addison's disease who was being kept in reasonable health by salt and D.O.C.A. The beneficial effects of testosterone propionate were so great that D.O.C.A. therapy was successfully discontinued. One of the actions of the androgen was to produce a dramatic fall in the serum potassium to levels as low as 0.7 m. eq. per litre. This did not, as might have been expected, lead to any of the signs which we associate with potassium deficiency in man, such as weakness or muscular paralysis. It would be interesting to know the effects of the prolonged administration of this drug upon the activity and well-being of organs such as the heart.

The blood banks have continued to stimulate work on the permeability of the red blood cell *in vitro*. Maizels (256) has published an extensive paper on the subject, and there is much information to be found in Davson & Danielli's book (239, 257). There is no doubt that the red cells lose their potassium and take up sodium when they are stored at low temperatures. Biochemists like to get their material into test tubes, and no one will deny the value of the blood banks or of the work done at them, but it is uncertain at the moment how much of it applies to the red cell as it circulates in the body. This, however, has been studied by a few people (239, 258) and most recently by Nordlander (259), who found that the cat's red cells lost potassium during their sojourn in the spleen. Much of this work in the future is likely to be carried out with radio elements, but for some purposes they will never be of any use.

The excretion of potassium has been studied in normal dogs (260), and in patients with severe renal insufficiency (79, 255). Winkler & Smith (260) found that, although the clearances rose rapidly with the level of potassium in the serum, they were never so great as the creatinine clearances (glomerular filtration rates), indicating that some potassium was always being reabsorbed. Keith *et al.* (255) have confirmed a fact, already generally recognised, that the excretion of potassium is usually well maintained even in the late

stages of chronic nephritis and nitrogen retention. These authors, and also Sanguinetti (79), have, however, noted that patients with nephritis excrete a test dose of potassium less readily than normal persons. Keith *et al.*, moreover, found that in normal persons who have been dehydrated by potassium salts, the potassium clearances may rise above the inulin clearances, suggesting that, even if potassium is always reabsorbed, excretion by the tubules must at times be even more active.

Sodium

Sodium chloride and potassium balances on five children have been carried out at two different levels of protein intake (261). At the higher intake of 4 gm. per kg. the children retained more chlorides and a little more sodium than they did at the lower intake of 3 gm. per kg. The authors attempted to calculate the sort of tissue being built up from the type of minerals retained, but it is very doubtful if such calculations are justified.

Radio-sodium appears to be absorbed so rapidly that it may be detected in human milk within twenty minutes of its ingestion (262). Radio-sodium and heavy water have been shown to be absorbed from stomach pouches in the dog, and the rate varies with the location of the pouch (263). It is clear from this, and also from the work of Hahn *et al.* (100) that the functions of the stomach must be seriously reconsidered so far as mineral metabolism is concerned (264). The absorption of radio-active phosphorus has been studied by Weissberger & Nasset (265), but these authors missed the possibilities of finding out about absorption in the stomach by giving the salt directly into the dog's duodenum. Adams *et al.* (266) have used the Cope pouch for a reinvestigation of the old problem of the effect of a dose of bicarbonate upon the secretion of hydrochloric acid. They conclude that it has a small stimulating effect for a few hours. The passage of radio-sodium and heavy water to and from the foetus has also been investigated (267, 268). The passage of water to and from the foetal guinea pig becomes much faster as gestation advances. It is always about 50 times more rapid than the transfer of sodium and 500 times greater than the amount incorporated in the growing tissue.

The insoluble sodium of bone has been studied by means of the radio-active isotope (269). It is inferred in this interesting paper that the sodium is adsorbed on to the crystals of hydroxy-apatite. Theoretically the amounts adsorbed should vary directly with the size and

inversely with the surface area of the crystals, and hence the relative amounts of insoluble sodium found in bone, dentine, and enamel have been explained. Further, these structures take up sodium in amounts which can be predicted from the Freundlich adsorption isotherm.

LITERATURE CITED

1. Kirsner, J. B., *J. Clin. Investigation,* **22,** 47–52 (1943)
2. Street, H. R., *J. Nutrition,* **24,** 111–19 (1942)
3. Lui, S. H., and Chu, H. I., *Medicine,* **22,** 103–62 (1943)
4. McCance, R. A., Widdowson, E. M., and Lehmann, H., *Biochem. J.,* **36,** 686–91 (1942)
5. Rau, V. S., and Murty, V. V. S., *Ann. Biochem. Exptl. Med.,* **2,** 87–100 (1942)
6. Fuhr, I., and Steenbock, H., *J. Biol. Chem.,* **147,** 65–75 (1943)
7. Widdowson, E. M., and McCance, R. A., *Lancet,* **1,** 230–32 (1943)
8. Mitra, K., and Mittra, H. C., *Indian J. Med. Research,* **30,** 299–307 (1942)
9. Mitra, K., and Mittra, H. C., *Indian J. Med. Research,* **31,** 41–43 (1943)
10. Khorana, M. L., Sarma, M. L., Seshagiri, R. P., and Giri, K. V., *Indian J. Med. Research,* **31,** 25–27 (1943)
11. Basu, K. P., and Ghosh, D., *Indian J. Med. Research,* **31,** 29–35 (1943)
12. Kelly, J., *J. Nutrition,* **25,** 303–8 (1943)
13. Basu, K. P., Basak, M. N., and De, H. N., *Indian J. Med. Research,* **30,** 309–13 (1942)
14. Basu, K. P., De, H. N., and Basak, M. N., *Indian J. Med. Research,* **30,** 417–22 (1942)
15. Basu, K. P., and Ghosh, D., *Indian J. Med. Research,* **31,** 37–39 (1943)
16. Breiter, H., Mills, R., Rutherford, E., Armstrong, W., and Outhouse, J., *J. Nutrition,* **23,** 1–9 (1942)
17. McCance, R. A., and Widdowson, E. M., *J. Physiol.,* **101,** 44–85 (1942)
18. Krebs, H. A., and Mellanby, K., *Biochem. J.,* **37,** 466–68 (1943)
19. Meyer, A. A., Oosthuizen, S. F., and Shapiro, H. A., *Lancet,* **2,** 639–40 (1942)

19a. Pringle, H., Reynolds, R. A., and Jessop, W. J. E., *J. Med. Assoc. Eire,* 67–68 (June, 1943)

20. McCance, R. A., and Widdowson, E. M., *J. Physiol.,* **101,** 304–13 (1942)
21. Wang, K., Liu, S. H., Chu, H. I., Yu, T. F., Chas, H. C., and Hsu, H. C., *Chinese Med. J.,* **61,** 61–72 (1942)
22. Cruickshank, E. W. H., Duckworth, J., Kosterlitz, H. W., and Warnock, G. M., *Nature,* **152,** 384–85 (1943)

23. Harrison, D. C., and Mellanby, E., *Biochem. J.*, **33**, 1660–80 (1939)
24. McCance, R. A., and Widdowson, E. M., *J. Physiol.*, **101**, 350–54 (1942)
25. Kosman, A. J., and Freeman, S., *Federation Proc.*, **2**, 27 (1943)
26. Nicolaysen, R., *Skand. Arch. Physiol.*, **69** (suppl.), 1–66 (1934)
27. Lehmann, H., and Pollak, L., *Biochem. J.*, **36**, 672–85 (1942)
28. Lehmann, H., *Nature*, **150**, 603 (1942)
29. Boyle, P. E., and Wesson, L. G., *Arch. Path.*, **36**, 243–52 (1943)
30. Sobel, A. E., Rockenmacher, M., and Kramer, B., *Federation Proc.*, **2**, 70 (1943)
31. Patwardhan, V. N., and Chitre, R. G., *Indian J. Med. Research*, **30**, 81–89 (1942)
32. Bruce, H. M., and Kassner, E. W., *Biochem. J.*, **37**, 105–9 (1943)
33. Klassen, K. P., and Curtis, G. M., *Arch. Internal Med.*, **71**, 78–94 (1943)
34. Morgan, A. F., and Shimotori, N., *J. Biol. Chem.*, **147**, 189–200 (1943)
35. Jones, J. H., *Federation Proc.*, **2**, 64 (1943)
36. Ritchie, B. V., *Med. J. Australia*, **1**, 331–36 (1942)
37. Bunkfeldt, R., and Steenbock, H., *J. Nutrition*, **25**, 479–90 (1943)
38. Booth, R. G., Henry, K. M., and Kon, S. K., *Biochem. J.*, **36**, 445–55 (1942)
39. Jones, J. H., *J. Biol. Chem.*, **142**, 557–67 (1942)
40. French, C. E., *J. Nutrition*, **23**, 375–84 (1942)
41. French, C. E., and Elliott, R. F., *J. Nutrition*, **25**, 17–22 (1943)
42. Nicolaysen, R., *Acta Physiol. Scand.*, **5**, 200–11 (1943)
43. Fairbanks, B. W., and Mitchell, H. H., *J. Nutrition*, **11**, 551–72 (1936)
44. Nicolaysen, R., *Acta Physiol. Scand.*, **5**, 215–18 (1943)
45. Nicolaysen, R., and Nordbø, R., *Acta Physiol. Scand.*, **5**, 212–14 (1943)
46. McCance, R. A., and Widdowson, E. M., *J. Physiol.*, **102**, 42–49 (1943)
47. Riddle, O., *Endocrinology*, **31**, 498–506 (1942)
48. Braude, R., Kon, S. K., and White, E. G., *J. Comp. Path. Therap.*, **53**, 161–89 (1943)
49. Braude, R., and Kon, S. K. (Unpublished data)
50. Craig, J. F., and Davies, G. O., *J. Comp. Path. Therap.*, **53**, 196–98 (1943)
51. Benjamin, H. R., Gordon, H. H., and Marples, E., *Am. J. Diseases Children*, **65**, 412–25 (1943)
52. Leverton, R. M., and Marsh, A. G., *Nebraska Agr. Expt. Sta. Research Bull.*, No. **125** (1942)
53. Robertson, J. D., *Nature*, **151**, 379–81 (1943)
54. Duckworth, J., and Warnock, G. M., *Nutrition Abstracts & Revs.*, **12**, 167–83 (1942)
55. Robinson, W. D., Janney, J. H., and Grande Covián, F., *J. Nutrition*, **24**, 557–84 (1942)
56. Wilson, D. C., and Widdowson, E. M., *Indian Med. Research Mem.*, No. **34** (1942)
57. Krishnan, K. N., *Indian J. Med. Research*, **30**, 589–602 (1942)
58. Gardner, W. U., and Pfeiffer, C. A., *Physiol. Revs.*, **23**, 139–65 (1943)
59. Mellanby, E., *J. Physiol.*, **101**, 408–31 (1942)
60. Bourne, G., *J. Physiol.*, **101**, 327–36 (1942)
61. Bourne, G., *Lancet*, **2**, 661–64 (1942)

62. Fraser, H. F., *Pub. Health Rept. Washington,* **57**, 959–67 (1942)
63. Fraser, H. F., and Topping, N. H., *Pub. Health Rept. Washington,* **57**, 968–73 (1942)
64. Tomlinson, T. H., Jr., *Pub. Health Rept. Washington,* **57**, 987–93 (1942)
65. Reid, M. E., *Physiol. Revs.,* **23**, 76–99 (1943)
66. Zucker, T. F., and Berg, B. N., *Proc. Soc. Exptl. Biol. Med.,* **53**, 34–36 (1943)
67. Boelter, M. D. D., and Greenberg, D. M., *J. Nutrition,* **26**, 105–21 (1943)
68. Deakins, M., and Looby, J., *Am. J. Obstet. Gynecol.,* **46**, 265–67 (1943)
69. Lund, A. P., and Armstrong, W. D., *J. Dental Research,* **21**, 513–18 (1942)
70. Fleming, A. M., *J. Obstet. Gynaecol. Brit. Empire,* **50**, 135–39 (1943)
71. McIntosh, J. F., *J. Clin. Investigation,* **21**, 755–62 (1942)
72. McIntosh, J. F., and Salter, R. W., *J. Clin. Investigation,* **21**, 771–74 (1942)
73. Inclan, A., *J. Am. Med. Assoc.,* **121**, 490–95 (1943)
74. Sheldon, J. H., *Proc. Roy. Soc. Med.,* **27**, 45–48 (1934)
75. Ghormley, R. K., *J. Am. Med. Assoc.,* **121**, 495 (1943)
76. Watchorn, E., and McCance, R. A., *Proc. Roy. Soc. Med.,* **27**, 101–2 (1934)
77. Follis, R. H., Jr., and Jackson, D. A., *Bull. Johns Hopkins Hosp.,* **72**, 232–41 (1943)
78. Falconer, M. A., and Cope, C. L., *Quart. J. Med.,* **11**, 121–54 (1942)
79. Sanguinetti, A. A., *Aplicaciones clínicas y dietéticas del metabolismo mineral* (Aniceto López, Buenos Aires, 1941)
80. Dine, R. F., and Lavietes, P. H., *J. Clin. Investigation,* **21**, 781–86 (1942)
81. Cope, C. L., and Wolff, B., *Biochem. J.,* **36**, 413–16 (1942)
82. Dubois, K. P., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.,* **147**, 699–704 (1943)
83. Greville, G. D., and Lehmann, H., *Nature,* **152**, 81–82 (1943)
84. Boyd, L. J., and Scherf, D., *Am. J. Med. Sci.,* **206**, 43–48 (1943)
85. Haury, V. G., *J. Lab. Clin. Med.,* **27**, 1361–75 (1942)
86. Williamson, M. B., and Gulick, A., *J. Cellular Comp. Physiol.,* **20**, 116–18 (1942)
87. Moore, C. V., Arrowsmith, W. R., Welch, J., and Minnich, V., *J. Clin. Investigation,* **18**, 553–80 (1939)
88. Widdowson, E. M., and McCance, R. A., *Lancet,* **1**, 588–91 (1942)
89. McCance, R. A., Edgecombe, C. N., and Widdowson, E. M., *Lancet,* **2**, 126–28 (1943)
90. Fuhr, I., and Steenbock, H., *J. Biol. Chem.,* **147**, 59–71 (1943)
91. Nakamura, F. I., and Mitchell, H. H., *J. Nutrition,* **25**, 39–48 (1943)
92. Pringle, W. J. S., and Moran, T., *J. Soc. Chem. Ind.,* **61**, 108–10 (1942)
93. Street, H. R., *J. Nutrition,* **26**, 187–95 (1943)
94. Freeman, S., and Ivy, A. C., *Am. J. Physiol.,* **137**, 706–9 (1942)
95. McCance, R. A., and Widdowson, E. M., *Lancet,* **2**, 680–84 (1937)
96. Brownlee, G., Bainbridge, H. W., and Thorp, R. H., *Quart. J. Pharm. Pharmacol.,* **15**, 148–65 (1942)
97. McCance, R. A., and Widdowson, E. M., *Nature,* **152**, 326–27 (1943)

98. Sandberg, M., Gross, H., and Holly, O. M., *Arch. Path.*, **33**, 834–44 (1942)
99. Gross, H., Sandberg, M., and Holly, O. M., *Am. J. Med. Sci.*, **204**, 201–5 (1942)
100. Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H., *J. Exptl. Med.*, **78**, 169–88 (1943)
101. Leverton, R. M., and Marsh, A. G., *J. Nutrition*, **23**, 229–38 (1942)
102. Johnston, F. A., and Roberts, L. F., *J. Nutrition*, **23**, 181–93 (1942)
103. Davidson, L. S. P., Donaldson, G. M. M., Dyar, M. J., Lindsay, S. T., and McSorley, J. G., *Brit. Med. J.*, **2**, 505–7 (1942)
104. Young, C. M., and McHenry, E. W., *Can. J. Pub. Health*, **34**, 367–70 (1943)
105. Fowler, W. M., and Barer, A. P., *J. Am. Med. Assoc.*, **118**, 421–27 (1942)
106. Barer, A. P., and Fowler, W. M., *Am. J. Med. Sci.*, **205**, 9–15 (1943)
107. Brøchner-Mortensen, K., *Acta Med. Scand.*, **113**, 43–57 (1943)
108. Moore, C. V., Minnich, V., Vilter, R. W., and Spies, T. D., *J. Am. Med. Assoc.*, **121**, 245–50 (1943)
109. Ross, J. F., and Chapin, M. A., *J. Clin. Investigation*, **21**, 640–41 (1942)
110. Deeny, J., Murdock, E. T., and Rogan, J. J., *Brit. Med. J.*, **1**, 721–23 (1943)
111. Casida, L. E., Meyer, R. K., and McShan, W. H., *Am. J. Physiol.*, **139**, 89–94 (1943)
112. Granick, S., *J. Biol. Chem.*, **146**, 451–61 (1942)
113. Granick, S., *J. Biol. Chem.*, **149**, 157–67 (1943)
114. Granick, S., *Proc. Soc. Exptl. Biol. Med.*, **53**, 255–56 (1943)
115. Granick, S., and Michaelis, L., *J. Biol. Chem.*, **147**, 91–97 (1943)
116. Michaelis, L., Coryell, C. D., and Granick, S., *J. Biol. Chem.*, **148**, 463–80 (1943)
117. Fankuchen, I., *J. Biol. Chem.*, **150**, 57–59 (1943)
118. Rothman, S., and Flesch, P., *Proc. Soc. Exptl. Biol. Med.*, **53**, 134–35 (1943)
119. Hamilton, J. G., *Radiology*, **39**, 541–72 (1942)
120. Greenberg, D. M., Copp, D. H., and Cuthbertson, E. M., *J. Biol. Chem.*, **147**, 749–56 (1943)
121. Kent, N. L., and McCance, R. A., *Biochem. J.*, **35**, 877–83 (1941)
122. Shils, M. E., and McCollum, E. V., *J. Am. Med. Assoc.*, **120**, 609–19 (1942)
123. Shils, M. E., and McCollum, E. V., *J. Nutrition*, **26**, 1–19 (1943)
124. Boyer, P. D., Shaw, J. H., and Phillips, P. H., *J. Biol. Chem.*, **143**, 417–25 (1942)
125. Combs, G. F., Norris, L. C., and Heuser, G. F., *J. Nutrition*, **23**, 131–40 (1942)
126. Rudra, M. N., *Ann. Biochem. Exptl. Med.*, **2**, 9–12 (1942)
127. Wessinger, G. D., and Weinmann, J. P., *Am. J. Physiol.*, **139**, 233–38 (1943)
128. Archibald, J. G., and Lindquist, H. G., *J. Dairy Sci.*, **26**, 325–30 (1943)
129. Dorrance, S. S., Thorn, G. W., Clinton, M., Edmonds, H. W., and Farber, S., *Am. J. Physiol.*, **139**, 399–405 (1943)

130. Martin, C. J. (Personal communication)
131. McCance, R. A., *Rev. Clin. Españ.*, **8**, 371–78 (1943)
132. McCance, R. A., and Widdowson, E. M., *Biochem. J.*, **36**, 692–96 (1942)
133. Sheline, G. E., Chaikoff, I. L., Jones, H. B., and Montgomery, M. L., *J. Biol. Chem.*, **147**, 409–14 (1943)
134. Sheline, G. E., Chaikoff, I. L., Jones, H. B., and Montgomery, M. L., *J. Biol. Chem.*, **149**, 139–51 (1943)
135. Montgomery, M. L., Sheline, G. E., and Chaikoff, I. L., *J. Exptl. Med.*, **78**, 151–59 (1943)
136. Murer, H. K., and Crandall, L. A., *J. Nutrition*, **23**, 249–58 (1942)
137. Fairhall, L. T., and Miller, J. W., *U.S. Pub. Health Repts.*, **56**, 1610–25 (1941)
138. Fairhall, L. T., and Miller, J. W., *U.S. Pub. Health Repts.*, **56**, 1641–50 (1941)
139. Kety, S. S., and Letonoff, T. V., *Am. J. Med. Sci.*, **205**, 406–14 (1943)
140. Letonoff, T. V., and Kety, S. S., *J. Pharmacol.*, **77**, 151–53 (1943)
141. Futch, C. E., *J. Am. Med. Assoc.*, **121**, 580–82 (1943)
142. White, W. B., Clifford, P. A., and Calvery, H. O., *J. Am. Vet. Med. Assoc.*, **102**, 292–93 (1943)
143. Bagchi, K. N., and Ganguly, H. D., *Indian J. Med. Research*, **27**, 777–91 (1940)
144. Bagchi, K. N., and Ganguly, H. D., *Ann. Biochem. Exptl. Med.*, **1**, 83–86 (1941)
145. Sachs, A., Levine, V. E., Hill, F. C., and Hughes, R., *Arch. Internal Med.*, **71**, 489–501 (1943)
146. Sheldon, J. H., *Haemochromatosis*, 205–23 (Oxford Univ. Press, 1935)
147. Finkel, A. J., Allee, W. C., and Garner, H. R., *J. Cellular Comp. Physiol.*, **20**, 179–87 (1942)
148. Drury, A., and Bradbury, J. T., *Am. J. Physiol.*, **139**, 135–38 (1943)
149. Ferguson, W. S., *Lancet*, **2**, 544 (1942)
150. Ferguson, W. S., Lewis, A. H., and Watson, S. J., *J. Agr. Sci.*, **33**, 44–51 (1943)
151. Lewis, A. H., *J. Agr. Sci.*, **33**, 52–57 (1943)
152. Lewis, A. H., *J. Agr. Sci.*, **33**, 58–63 (1943)
153. Ferguson, W. S., *J. Agr. Sci.*, **33**, 116–18 (1943)
154. McClendon, J. F., Foster, W. C., and Supplee, G. C., *Arch. Biochem.*, **1**, 51–57 (1943)
155. McClendon, J. F., and Foster, W. C., *Federation Proc.*, **2**, 34 (1943)
156. Deatherage, C. F., *J. Dental Research*, **22**, 129–37 (1943)
157. Deatherage, C. F., *J. Dental Research*, **22**, 173–80 (1943)
158. Irving, J. T., *S. African Dental J.*, **15**, 278–82 (1941)
159. McClure, F. J., *J. Dental Research*, **22**, 37–43 (1943)
160. Irving, J. T., *Nature*, **151**, 363 (1943)
161. McClendon, J. F., and Foster, W. C., *Federation Proc.*, **2**, 33–34 (1943)
162. Linsman, J. F., and McMurray, C. A., *Radiology*, **40**, 474–84 (1943)
163. Kemp, F. H., Murray, M. M., and Wilson, D. C., *Lancet*, **2**, 93–97 (1942)
164. Editorial, *Lancet*, **2**, 242 (1943)

165. Spira, L., *J. Hyg.*, **43**, 69–71 (1943)
166. Bromehead, C. N., Murray, M. M., and Wilson, D. C., *Lancet*, **1**, 490–91 (1943)
167. Spira, L., and Grimbleby, F. H., *J. Hyg.*, **43**, 142–45 (1943)
168. Murray, M. M., and Wilson, D. C., *Lancet*, **1**, 818 (1943)
169. Lidbeck, W. L., and Beeman, J. A., *J. Am. Med. Assoc.*, **121**, 826–27 (1943)
170. Astwood, E. B., *J. Am. Med. Assoc.*, **122**, 78–81 (1943)
171. Himsworth, H. P., *Lancet*, **2**, 465 (1943)
172. Hamilton, J. G., and Lawrence, J. H., *J. Clin. Investigation*, **21**, 624 (1942)
173. Carter, G. S., Mann, F. G., Harley-Mason, J., and Jenkins, G. N., *Nature*, **151**, 728–30 (1943)
174. Sharpless, G. R., and Anthony, E. K., *J. Nutrition*, **25**, 239–43 (1943)
175. Bruger, M., and Member, S., *J. Biol. Chem.*, **148**, 77–83 (1943)
176. Man, E. B., Smirnow, R. E., Gildea, E. F., and Peters, J. P., *J. Clin. Investigation*, **21**, 773–80 (1942)
177. Morton, M. E., and Chaikoff, I. L., *J. Biol. Chem.*, **147**, 1–9 (1943)
178. Franklin, A. L., and Chaikoff, I. L., *J. Biol. Chem.*, **148**, 719–20 (1943)
179. Leblond, C. P., Peacock, W. C., Gross, J., and Evans, R. D., *Federation Proc.*, **2**, 29 (1943)
180. Morton, M. E., Chaikoff, I. L., Reinhardt, W. O., and Anderson, E., *J. Biol. Chem.*, **147**, 757–69 (1943)
181. Leblond, C. P., Gross, J., Peacock, W. C., and Evans, R. D., *Federation Proc.*, **2**, 28–29 (1943)
182. Bruger, M., and Member, S., *Am. J. Physiol.*, **139**, 212–16 (1943)
183. Flexner, J., Bruger, M., and Member, S., *Federation Proc.*, **1**, 109 (1942)
184. Bagchi, K. N., and Ganguly, H. D., *Indian Med. Gaz.*, **76**, 720–28 (1941)
185. Kent, N. L., and McCance, R. A., *Biochem. J.*, **35**, 837–44 (1941)
186. Block, W. D., and Wells, G. S., *J. Clin. Investigation*, **21**, 645 (1942)
187. Seifter, J., and Rambousek, E. S., *J. Lab. Clin. Med.*, **28**, 1344–48 (1943)
188. Fay, M., Andersch, M. A., and Behrmann, V. G., *J. Biol. Chem.*, **144**, 383–92 (1942)
189. Weinmann, J. P., *J. Dental Research*, **21**, 497–504 (1942)
190. Treadwell, A. de G., Low-Beer, V. A., Friedwell, H. L., and Lawrence, J. H., *Am. J. Med. Sci.*, **204**, 521–30 (1942)
191. Pecher, C., *Univ. Calif. Pub. Pharmacol.*, **2**, 117–49 (1942)
192. Fitz-Hugh, T., and Hodes, P. J., *Am. J. Med. Sci.*, **204**, 662–65 (1942)
193. Youmans, G. P., and Delves, E., *J. Bact.*, **44**, 127–36 (1942)
194. Beaser, S. B., Segel, A., and Vandam, L., *J. Clin. Investigation*, **21**, 447–54 (1942)
195. Smythe, C. V., *Arch. Biochem.*, **2**, 259–68 (1943)
196. Tarr, H. L. A., and Carter, N. M., *J. Fish. Res. Bd. Can.*, **6**, 63–73 (1942)
197. Ashworth, C. T., Muirhead, E. E., Thomas, O. F., and Hill, J. M., *Am. J. Physiol.*, **139**, 255–60 (1943)
198. Winkler, A. W., Elkington, J. R., and Eisenman, A. J., *Am. J. Physiol.*, **139**, 239–46 (1943)
199. Wilde, W. S., *Science*, **98**, 202–3 (1943)

200. Talbot, N. B., Butler, A. M., and MacLachlan, E. A., *J. Clin. Investigation*, **22**, 583–93 (1943)
201. Crismon, J. M., Crismon, C. S., Calabresi, M., and Darrow, D. C., *Am. J. Physiol.*, **139**, 667–74 (1943)
202. Darrow, D. C., and Miller, H. C., *J. Clin. Investigation*, **21**, 601–12 (1942)
203. Harkness, D. M., Muntwyler, E., Mautz, F. R., and Mellors, R. C., *J. Lab. Clin. Med.*, **28**, 307–13 (1942)
204. Childs, A., and Eichelberger, L., *Am. J. Physiol.*, **137**, 384–91 (1942)
205. Barbour, H. G., McKay, E. A., and Griffith, W. P., *Federation Proc.*, **2**, 2 (1943)
206. Kirsner, J. B., Palmer, W. L., and Knowlton, K., *J. Clin. Investigation*, **22**, 95–102 (1943)
207. McCance, R. A., and Widdowson, E. M., *Proc. Roy. Soc. (London) B*, **120**, 228–39 (1936)
208. McCance, R. A., and Widdowson, E. M., *J. Physiol.*, **91**, 222–31 (1937)
209. Kirsner, J. B., and Palmer, W. L., *Arch. Internal Med.*, **71**, 415–21 (1943)
210. Cohn, C., and Soskin, S., *Am. J. Physiol.*, **139**, 80–83 (1943)
211. Cohn, C., Levine, R., and Soskin, S., *Am. J. Physiol.*, **139**, 84–88 (1943)
212. Elkington, J. R., and Taffel, M., *J. Clin. Investigation*, **21**, 787–94 (1942)
213. Ladell, W. S. S., *Lancet*, **2**, 441–44 (1943)
214. Black, D. A. K., McCance, R. A., and Young, W. F., *J. Physiol.* (In press)
215. Nadal, J. W., Pedersen, S., and Maddock, W. G., *J. Clin. Investigation*, **20**, 691–703 (1941)
216. Black, D. A. K., McCance, R. A., and Young, W. F., *Nature*, **150**, 461 (1942)
217. McCance, R. A., and Young, W. F., *J. Physiol.* (In press)
218. Sayers, G., Sayers, M., and Orten, J. M., *J. Nutrition*, **26**, 139–51 (1943)
219. McCance, R. A., *Biochem. J.*, **31**, 1276–77 (1937)
220. Emerson, K., Curmen, E. C., Mirick, G. S., and Ziegler, J. E., *J. Clin. Investigation*, **22**, 695–97 (1943)
221. Hare, K., and Hare, R. S., *Federation Proc.*, **2**, 19 (1943)
222. Farnsworth, E. B., and Barker, M. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 74–75 (1943)
223. Eggleton, M. G., *J. Physiol.*, **102**, 140–54 (1943)
224. Prunty, F. T. G., and Macoun, S. J. R., *Brit. J. Exptl. Path.*, **24**, 22–25 (1943)
225. Snelling, C. E., *J. Pediat.*, **22**, 559–64 (1943)
226. Young, W. F., and McCance, R. A., *Arch. Diseases Childhood*, **17**, 65–81 (1942)
227. Aldridge, A. G. V., *Arch. Diseases Childhood*, **16**, 81–94, 182–205 (1941)
228. Field, J. B., Elvehjem, C. A., and Juday, C., *J. Biol. Chem.*, **148**, 261–69 (1943)
229. Griffiths, J. T., and Tauber, O. E., *J. Gen. Physiol.*, **26**, 541–58 (1943)
230. Rubinstein, D. L., Lwowa, W., and Burlakowa, H., *Biochem. Z.*, **278**, 418–27 (1935)
231. Leibowitz, J., and Kupermintz, N., *Nature*, **150**, 233 (1942)

232. Rothstein, A., and Haege, L., *Federation Proc.*, **2**, 42–43 (1943)
233. Farmer, S. N., and Jones, D. A., *Nature*, **150**, 768–69 (1942)
234. Conway, E. J., *Nature*, **150**, 461–62 (1942)
235. Scott, G. T., *J. Cellular Comp. Physiol.*, **21**, 327–38 (1943)
236. Reilly, D., and Curtin, T. P., *Biochem. J.*, **37**, 36–39 (1943)
237. Spangler, C. D., and Winslow, C. E., *J. Bact.*, **45**, 373–84 (1943)
238. Krogh, A., *Osmotic regulation in aquatic animals* (Cambridge University Press, 1939)
239. Davson, H., and Danielli, J. F., *The permeability of natural membranes* (Cambridge University Press, 1943)
240. Hevesy, G., *Acta Physiol. Scand.*, **3**, 123–31 (1942)
241. Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.*, **149**, 529–42 (1943)
242. Fenn, W. O., Mullins, L. J., Adler, T. K., and Haege, L., *Federation Proc.*, **2**, 11–12 (1943)
243. Rowell, R. E., *Brit. Med. J.*, **2**, 483–84 (1943)
244. Winkler, A. W., and Hoff, H. E., *Am. J. Physiol.*, **139**, 686–92 (1943)
245. Mylon, E., Winternitz, M. C., and Sütö-Nagy, G. J., *Am. J. Physiol.*, **139**, 299–306 (1943)
246. Manery, J. F., and Solandt, D. Y., *Am. J. Physiol.*, **138**, 499–511 (1942–43)
247. Lyman, C. P., *Am. J. Physiol.*, **137**, 392–95 (1942)
248. Hiatt, E. P., *Am. J. Physiol.*, **139**, 45–48 (1943)
249. Felter, D., Coombs, H. C., and Pike, F. H., *Federation Proc.*, **2**, 12 (1943)
250. Durlacher, S. H., and Darrow, D. C., *Am. J. Physiol.*, **136**, 577–83 (1942)
251. Durlacher, S. H., Darrow, D. C., and Winternitz, M. C., *Am. J. Physiol.*, **136**, 346–49 (1942)
252. Follis, R. H., *Bull. Johns Hopkins Hosp.*, **71**, 235–41 (1942)
253. Follis, R. H., *Am. J. Physiol.*, **138**, 246–50 (1942–43)
254. Follis, R. H., *Proc. Soc. Exptl. Biol. Med.*, **51**, 71–72 (1942)
255. Keith, N. M., King, H. E., and Osterberg, A. E., *Arch. Internal Med.*, **71**, 675–701 (1943)
256. Maizels, M., *Quart. J. Exptl. Physiol.*, **32**, 143–81 (1943)
257. Davson, H., *J. Physiol.*, **101**, 265–83 (1942)
258. McCance, R. A., *Biochem. J.*, **31**, 1278–84 (1937)
259. Nordlander, N. B., *Acta Physiol. Scand.*, **4**, 323–29 (1942)
260. Winkler, A. W., and Smith, P. K., *Am. J. Physiol.*, **138**, 94–103 (1942–43)
261. Hawks, J. E., Bray, M. M., Hartt, S., Whittlemore, M. B., and Dye, M., *J. Nutrition*, **24**, 437–48 (1942)
262. Pommerenke, W. T., and Hahn, P. F., *Proc. Soc. Exptl. Biol. Med.*, **52**, 223–24 (1943)
263. Cope, O., Cohn, W. E., and Brenizer, A. G., *J. Clin. Investigation*, **22**, 103–10 (1943)
264. Gudiksen, E., *Acta Physiol. Scand.*, **5**, 39–54 (1943)
265. Weissberger, L. H., and Nasset, E. S., *Am. J. Physiol.*, **138**, 149–55 (1942–43)

266. Adams, W. L., Welch, C. S., and Clark, B. B., *Am. J. Physiol.*, **139**, 356–63 (1943)
267. Gellhorn, A., and Flexner, L. B., *Am. J. Physiol.*, **136**, 750–56 (1942)
268. Flexner, L. B., and Gellhorn, A., *Am. J. Physiol.*, **136**, 757–61 (1942)
269. Hodge, H. C., Koss, W. F., Ginn, J. T., Falkenheim, M., Gavett, E., Fowler, R. C., Thomas, I., Bonner, J. F., and Dessauer, G., *J. Biol. Chem.*, **148**, 321–31 (1943)

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THE CHEMISTRY OF THE HORMONES

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Only a comparatively small number of papers dealing with the chemistry of the different hormones has appeared during the year of 1943. Apparently the present preoccupation with more immediately practical problems has temporarily interrupted research in this field. Foreign journals have been only partially accessible. The chemistry of the steroid hormones and the excretion of these principles have only been briefly referred to since these subjects will be discussed in another chapter by Professor F. C. Koch. The chemistry of plant growth substances is reviewed in this volume by Dr. I. van Overbeek. Since the chemistry of the hormones of the gastrointestinal tract and of antihormones has not recently been reviewed, the more important literature prior to 1943 has been included. I wish to tender my apology to the authors whose papers may not have been reviewed, and for the unavoidable oversights and possible misinterpretation of results in the subjects treated.

Pituitary

Lactogenic hormone.—Li, Simpson & Evans (1) have described a procedure for the isolation of the pituitary lactogenic hormone from sheep or ox glands. The preparation was found to be chemically pure by electrophoretic, solubility, and diffusion tests and to have an activity of 25 to 30 I.U. per mg. Lactogenic preparations of similar potency have been obtained by Schwenk, Fleischer & Tolksdorf (2). According to these investigators treatment of fresh, macerated pituitaries with chloroform separates the pituitary proteins into two fractions, an aqueous solution, containing among other active principles the gonadotrophins and thyrotrophin, and a solid gel, containing prolactin and adrenocorticotrophin. The lactogenic hormone is obtained from the chloroform gel by extraction with acid methanol and sodium chloride fractionation. Fleischer (3) has shown that prolactin is highly soluble, at a pH below its isoelectric point, in 99.8 per cent methanol and in 95 per cent ethanol. This behavior, however, is shown by a large number of the proteins from fresh pituitary glands. Li (4) from diffu-

sion and viscosity measurements of a highly purified lactogenic hormone preparation arrived at a molecular weight of 22,000 for this principle. The methionine and cystine content of highly purified prolactin was found by Li (5) to be 4.31 and 3.11 per cent, respectively. Within limits of experimental error, methionine and cystine contents account for the total sulfur in the hormones. Further studies on the specificity of the local crop-sac test for prolactin in pigeons have been reported by Lahr, Bates & Riddle (6).

Trentin, Lewis, Bergman & Turner (7) have reported experiments which indicate that the mammary duct growth stimulating factor, designated as mammogen I, is present in the protein fraction of the anterior pituitary rather than in the fraction extracted by lipid solvents which previous work by Lewis & Turner (8) had indicated.

Thyrotrophic hormone. — A procedure for obtaining from beef pituitaries highly potent thyrotrophic preparations, which behaved like a homogeneous substance in the Tiselius apparatus and in the ultra-centrifuge, has been briefly described by Ciereszko & White (9).

Adrenocorticotrophic hormone. — Methods for obtaining highly purified adrenocorticotrophic anterior pituitary preparations have been simultaneously published by different laboratories (10, 11, 12). The procedure of Li, Evans & Simpson (10) starting from sheep glands consists mainly of salt fractionation, while the procedure of Sayers, White & Long (11) involves chiefly isoelectric precipitation of hog gland extract. Neufeld (12) has adopted a procedure similar to the two outlined. The preparations obtained from different sources by the Berkeley and New Haven groups seem to be similar if not identical as judged from their physical chemical properties as found in the two laboratories (10, 11). Electrophoretic, sedimentation, and solubility experiments indicate the preparations to be homogeneous. Biological tests show them to be free of other anterior hypophyseal hormones. Molecular weight determinations gave a value of 20,000 (13), and the isoelectric point was found to be between pH 4.7 and 4.8. Li, *et al.* (10) showed that adrenocorticotrophic activity is destroyed by trichloroacetic acid and by tryptic digestion but shows a marked stability to peptic digestion. Simpson, Evans & Li (15) have proposed two methods for standardizing the adrenocorticotrophic potency of pituitary extracts. The units are defined as (*a*) the total dose in milligrams necessary to cause beginning adrenal repair (redistribution of cortical lipid) in female rats twenty-six to twenty-eight days old at hypophysectomy and fourteen days post-operative at the beginning of

the four day injection period, and (*b*) the daily dose in milligrams necessary to maintain the pre-operative adrenal weight for fifteen days in male rats hypophysectomized at forty days of age. Tyslowitz (14) has reported that dialysates of hog pituitary extract contain a factor which increases the size of the adrenals in hypophysectomized rats.

Growth and metabolic hormones.—Marx, Simpson & Evans (16) described a procedure for the preparation and purification of growth hormone from beef anterior pituitary lobes. It consists of the following five steps: desiccation of the gland tissue with acetone, extraction with calcium hydroxide, precipitation with ammonium sulfate, treatment with cysteine, and further fractionation by pH variation. The final product had a potency of approximately 130 growth hormone units per mg. (hypophysectomized rat units). Compared with the calcium hydroxide extract, this represents approximately a 16-fold increase in potency. Evans and his associates (17) have described a new method for the bioassay of the pituitary growth hormone. This method is based upon the observation that hypophysectomy causes regressive changes, which can be reversed with growth hormone, at the proximal end of the tibia in the immature rat. The increase in width of the cartilage observed during the administration of growth hormone is employed as the criterion of assay. Antagonism has been demonstrated on simultaneous administration of growth hormone and of adrenocorticotrophic hormone (18), while synergism was obtained when the growth hormone was given in combination with purified thyrotrophic pituitary hormone (19). According to Herring & Evans (20) administration of either crude anterior pituitary extracts or of highly purified growth-promoting preparations were most potent in producing an increase in muscle glycogen. Administration of the adrenocorticotrophic principle showed slight or no activity during the one-day test in which growth hormone preparations were highly effective. Purified growth hormone preparations of the anterior pituitary produce a marked increase in glucose excretion in the sucrose-fed, partially depancreatized rat (21). Fraenkel-Conrat, Simpson & Evans (22) reported that hypophysectomy leads to a marked decrease in liver arginase activity. This process could be reversed by the administration of adrenocorticotrophic hormone. This pituitary principle also increased the arginase activity of the livers of normal rats. In contrast, growth hormone was found to decrease the arginase activity in hypophysectomized and normal rats.

There is as yet no definite proof that the anterior pituitary secretes

a specific ketogenic principle. The findings of Shipley (23) indicate that growth and ketogenic effects are due to two separate principles which apparently have similar molecular size (24). Gray (25) found that the ketogenic activity is distributed fairly evenly among the albumin, globulin, and pseudoglobulin fractions of pH 5.5 soluble extracts of the anterior pituitary.

Pars intermedia and neuralis.—It is still an open question whether the pharmacological activities of the posterior lobe are due to a single protein molecule having multiple activities or to different molecules of smaller size which may or may not be integral parts of a single molecule in the gland itself. Smith (26) made a comparison of the official and the chicken methods for the oxytocic bioassay of posterior pituitary preparations. He found that the chicken method has many practical advantages over the official method. A procedure for the separation of the pressor and oxytocic hormones by the use of mixtures of acetone with methanol or ethanol has been described (27).

From posterior lobe pituitary powder potent melanophore-expanding preparations have been obtained having 85,000 Landgrebe-Waring units per mg. and less than 1 I.U. of pressor activity (28). Abramowitz, Papandrea & Hisaw (29) have described a method for the purification of intermedin. The final preparation represents a purification of 200-fold over that of the original pituitary tissue. Chen & Geiling (30) observed an increase in potentiation of the melanophore hormone with change in pH from low to high values and a difference in pharmacological properties between the "potentiated" and the "unpotentiated" substance. They believe that potentiation of the hormone is due to an effect on the molecule by changes in pH. For detailed information on the recent advances in the chemistry of the various pituitary hormones the reader is referred to a review published by The New York Academy of Sciences (31).

Gonadotrophic Hormones

Anterior pituitary.—McShan & Meyer (32) have published an additional procedure for the preparation and purification of sheep pituitary gonadotrophin. Addition of hemin to pituitary gonadotrophic extracts produces augmentation of the physiological effect in the normal male (33) as well as in the normal female rat (34).

Urinary gonadotrophin.—Katzman, *et al.* (35) have described a new method for the preparation and purification of chorionic gonado-

trophin of pregnancy urine. This method is based on the chromatographic adsorption of the active principle on permutit and its elution with an alcoholic solution of ammonium acetate. The hormone is precipitated from the eluate by increasing the concentration of alcohol. Preparations assaying 8,500 I.U. per mg. have been obtained in this way. Material of similar potency of chorionic gonadotrophin has been previously described by Gurin and his associates (36). Bischoff (37) found that more than 90 per cent of the physiological activity of chorionic gonadotrophin is destroyed on treatment with phenylisocyanate under mild conditions of reaction. Salmon & Hamblen (38) reported that the amount of gonadotrophin recovered from the urine of rabbits during the first ninety-six hours following the administration of chorionic gonadotrophin was 5.7 per cent of the total amount injected. No gonadotrophin was found in the urine after ninety-six hours. Jones, Gey & Gey (39) demonstrated that placental cells maintained in continuous culture produce chorionic gonadotrophin and may retain this capacity as long as six months. It has not been possible to demonstrate that placental cells can produce estrogen under similar circumstances.

Only a few studies have been reported on the gonadotrophins found in the urine of normal men and of normally menstruating or menopausal women. Leathem & Mills (40) found that gonadotrophic extracts of normal male urine restores and maintains spermatogenesis in hypophysectomized rats and causes a definite increase in testis weight in the hypophysectomized immature animal. These results show that male urine gonadotrophin extracts give predominantly a gamotogenic effect. Levin (41) reported that tannic acid precipitation recovers more gonadotrophic activity from the urine of normal men than does alcohol precipitation. Evans & Gorbman (42) described a modified alcoholic precipitation method for the preparation of gonadotrophic concentrates from normal male urine.

Pregnant mare serum gonadotrophin (equine).—Rimington & Rowlands (43) have briefly described a method of purification of pregnant mare serum gonadotrophic preparations by fractional alcohol precipitation at varying pH. These investigators have obtained preparations assaying about 12,000 I.U. per mg.

The question is still open whether the gonadotrophic effect of pregnant mare serum is exerted by two distinct separate principles, one specifically affecting the interstitial tissue of the ovary and Leydig tissue of the testis, and the second causing follicular growth in the

female and germinal tissue development in the male, or whether it is the property of a single individual principle. The observation of Kupperman, Meyer & McShan (44) that antifollicle-stimulating hormone serum could selectively neutralize the follicle-stimulating activity of pregnant mare serum preparations without inhibiting the luteinizing action would seem to indicate that the equine gonadotrophic effect is elicited by two distinct principles. The finding of Jensen, Hauschildt & Evans (45) that an augmentative effect could be observed when pregnant mare serum gonadotrophin was administered in combination with a pituitary follicle-stimulating preparation, while the addition of chorionic gonadotrophin did not potentiate the gonadotrophic response of equine, indicates that the gonadotrophic effect of pregnant mare serum is mainly a luteinizing response. A comprehensive study of the effect of equine gonadotrophin on the testes of hypophysectomized monkeys has been carried out by Smith and his associates (46).

Biological properties.—It is generally accepted that pregnant mare serum and chorionic gonadotrophin evoke different biological responses in appropriate test animals. It is also evident that the active gonadotrophic substance or substances in the urine of castrate and post-menopausal women and of normal men differ among themselves and from the chorionic principles (human chorionic gonadotrophin and equine). The present status of gonadotrophic therapy in gynecological practice has recently been reviewed by Davis & Hellbaum (47).

Biological assay.—It is generally recognized that reliable results on the specific biological response of gonadotrophic preparations can best be obtained in hypophysectomized animals. Assays in normal animals may lead to erroneous interpretation of the physiological effects observed. Modified procedures have been published for assaying the gonadotrophic activity of anterior pituitary follicle-stimulating hormone (48, 49) and of chorionic gonadotrophin (50, 51, 52).

Thyroid

That the thyroid gland is not essential for the synthesis of thyroxine has been demonstrated by the isolation of radioactive thyroxine following the administration of radioactive iodine to rats which were deprived of all thyroid tissue (53). The *in vitro* conversion of iodine to diiodotyrosine and thyroxine by surviving slices of thyroid gland excised from sheep, dog, and rat has been demonstrated by Morton &

Chaikoff (54) with the aid of radioactive iodine. Schachner, Franklin & Chaikoff (55) found that cyanide, azide, sulfide, and carbon monoxide inhibits the formation of diiodotyrosine and thyroxine from inorganic iodide by thyroid tissue. These investigators believe that the formation of both diiodotyrosine and thyroxine by the thyroid gland is linked with aerobic oxidations in which the cytochrome oxidase system is involved. Franklin & Chaikoff (56) showed that sulfanilamide has an inhibitory effect on the conversion of inorganic iodine to both diiodotyrosine and thyroxine. This latter observation is in agreement with recent observations that the administration of certain sulfonamides produces enlargement and hyperemia of the thyroid gland in the rat, coincident with a state of hypothyroidism (57, 58). It has been suggested that these compounds act on the synthesis of thyroxine. Thiourea and uracil have been reported to be effective in thyrotoxicosis in man (59, 60).

According to Turner and his associates (61, 62) the critical factors in the chemical formation of artificial thyroproteins with high thyroidal activity are the degree of iodination of the protein, the pH of the reaction medium, and the temperature at which the iodination and incubation processes are carried out. Lautenschläger & Bockmühl (63) have studied the formation of *dl*-thyroxine by iodination of different proteins under various experimental conditions. Reineke & Turner (64) have obtained about 400 mg. of crystalline thyroxine of good purity from 100 gm. of iodinated casein. The same authors also reported that direct hydrolysis of iodinated casein with a sulfuric acid-butyl alcohol mixture gave a yield of approximately 0.1 per cent of crystalline *l*-thyroxine (65).

Carter, *et al.* (66) reported results which have led these investigators to the conclusion that the basal metabolic rate is normally controlled in the rat not by the concentration of the thyroid secretion in the internal medium alone but by the interaction between this secretion and an anti-thyroid substance which they have identified as paraxanthine (1,7-dimethylxanthine). These observations may throw new light on the formation of antihormones. Paraxanthine may be effective in the treatment of thyrotoxicosis in man. Rawson, Sterne & Aub (67) observed that the thyroid stimulating effect of a pituitary extract is destroyed following its exposure to normal thyroid tissue. For detailed information on the new approaches to the physiology of the thyroid the reader is referred to a recent comprehensive review by Means (68).

Insulin

No additional progress on the chemistry of insulin has been made during the period under review. A method for detecting the presence of insulin in urine has been described and investigated by Cutting (69). Lukens and his associates (70, 71) reported that treatment of partially depancreatized cats, made diabetic by injection of anterior pituitary extract, with either insulin or phlorizin is followed by morphological restoration and functional recovery of the islet tissue in the pancreas if the treatment is begun within the first three months. After three months of the diabetic state, when atrophy of the pancreatic islet tissue had developed, there was no recovery after the administration of either insulin or phlorizin. Further reports on the clinical value of various insulin preparations in the treatment of diabetes mellitus have appeared (72 to 75). Reference should be made to the interesting observation of Dunn & McLetchie (76) that alloxan, in doses of 300 to 400 mg. per kg. body weight, introduced subcutaneously or intramuscularly in rats, usually caused severe acute damage of the islet tissue in the pancreas. Glycosuria developed in rats which survived the treatment, and some animals exhibited practically all the signs and symptoms of severe diabetes mellitus. The glycosuria could be abolished by injections of insulin. These findings have been confirmed by Bailey & Bailey (77) for the rabbit, and by Goldner & Gomori (78) for the dog.

Adrenal Cortex

A comprehensive review on the chemistry of the hormones of the adrenal cortex has been published by Reichstein & Shoppee (79). The partial synthesis of dehydrocorticosterone has recently been accomplished by Lardon & Reichstein (80, 81, 82). The synthetic product was found to be identical with the natural substance. Shoppee & Reichstein (83) found that anhydro-corticosterone acetate, obtained on treatment of corticosterone acetate with acid, is about two to three times more potent than desoxycorticosterone acetate in the Everse-de Fremery test. Both compounds show about the same activity when tested for life maintenance in adrenalectomized rats. Segaloff & Nelson (84) reported that Δ^5-pregnene-3-ol-20-one-21-ol-acetate falls between progesterone and desoxycorticosterone acetate in its ability to produce growth and to lengthen the survival time of adrenalectomized, immature male rats. Kuizenga, *et al.* (85) observed that hog adrenal

cortical extract was considerably more potent by both the adrenalectomized rat survival test and by the muscle contraction test, than beef and sheep adrenal cortical extracts. These investigators expressed the belief that the higher activity of hog extract may be due to the increased amount of C_{11} oxygenated steroids present.

Reinecke & Kendall (86, 87) have proposed a method for the bioassay of adrenal cortical extracts based on the amount of glycogen deposited in the liver of a fasting, adrenalectomized rat after injection of the test solution. Bergman & Klein (88) reported on their experience with the method of Reinecke & Kendall (86, 87). They found that in fasted, adrenalectomized male rats, injected with adrenal cortical extracts, the total amounts of glycogen stored in the liver for a given dose of extract is independent of the body weight, at least in the range of 130 to 340 gm. The total amount of glycogen deposited is likewise independent of the weight of the liver. According to Rogoff (89) the only reliable method of assay by which potency and purity of adrenal cortical extracts can be assured is standardization based upon survival and health of bilaterally adrenalectomized male dogs. Ingle (90) has published further details of his method of biological assay of cortical hormones based upon the performance of work by adrenalectomized rats.

Fraenkel-Conrat, Simpson & Evans (22, 91) have shown that adrenalectomy causes a marked decrease of the arginase activity of rat livers. Administration of those adrenal cortical principles containing an oxygen at C_{11} has been found to produce an increase of arginase in the liver in normal, hypophysectomized, and adrenalectomized animals. Fraenkel-Conrat (92) found desoxycorticosterone acetate to be beneficial to adrenalectomized rats upon oral administration; i.e., mixed with food and/or drinking water. Dosages of 0.09 to 0.33 mg. daily favored survival and growth in such rats on a salt-free diet. Griffiths (93) claims that the anti-insulin effect of cortical extracts is manifested only when the insulin is administered by subcutaneous injection, and that there is no antagonistic effect of cortical extract on intravenously injected insulin. Griffiths expressed the belief that the cortical hormones inhibit the rate of absorption of insulin from the subcutaneous tissues. This explanation is in disagreement with the one presented by Jensen and collaborators (94) that the anti-insulin effect of certain adrenal cortical principles is due to the ability of these substances to increase the amount of liver glycogen. One possible explanation for the finding of Griffiths is that intravenously injected insulin, by virtue

of its more rapid action, is the equivalent of a larger subcutaneous dose. Concerning the present status of our knowledge of the physiology of the adrenal cortex the reader is referred to a recent comprehensive review by Ingle (95).

The excretion of cortical hormones having an oxygen at C_{11} and an α,β unsaturated ketone in ring A is indicated by the recent demonstration of different investigators that extracts of urine cause the deposition of liver glycogen in addition to maintaining the life of adrenalectomized rats (96, 97). Hoffman, Kazmin & Browne (98) found that desoxycorticosterone when administered to rabbits is excreted as pregnanediol glucuronide.

Parathyroid

Evidence has been presented by Carnes, Osebold & Stoerk (99) that the function of the parathyroid glands is unimpaired in the young mature male rat deprived of its hypophysis. The hypophysectomized animal maintained its serum calcium and inorganic phosphate concentration within normal limits. In contrast, the hypophysectomized-parathyroidectomized animal under the same conditions suffered a profound fall in serum calcium and rise in serum phosphate, quite like the parathyroidectomized control. These results cast a serious doubt on the existence of a direct physiological regulation of the parathyroid gland by the hypophysis.

Ross & Wood (100) obtained parathyroid preparations, assaying 250 to 350 U.S.P. units per mg. of nitrogen, by fractional precipitation with ammonium sulfate at pH 6.0, and by adsorption on benzoic acid. Pepsin completely inactivated the principle at pH 4.1. Acetylation of the parathyroid hormone with ketene is accompanied by complete biological inactivation (101).

In vitro studies on the influence of parathyroid hormone on kidney phosphatase indicate that activation occurs; however, the effect is not specific since egg albumin and serum albumin were found to produce a similar response (102). Apparently the hormone of the parathyroid does not act on the bones through the intermediary of the kidney (103, 104), and the serum calcium elevating activity of the parathyroid hormone is also independent of the kidney (105).

Hormones of the Gastrointestinal Tract

This section deals with recent contributions to the chemical studies of substances extracted from the various sections of the alimentary canal.

Gastrin.—The question whether gastrin, the principle present in extracts of the pyloric mucosa and causing the gastric gland to secrete, is truly a hormone and whether it is or is not histamine, has been recently reviewed by Ivy (106). The production of histamine-free extracts of pyloric mucosa has been reported by Komarov (107, 108, 109). These extracts were found not to depress the blood pressure and to contain two active principles, separable by fractional precipitation, one of which stimulates the gastric glands and the other the external secretion of the pancreas. The secretin fraction of the extracts appeared to be closely similar to that obtained from the duodenal mucosa. The extracts were reported to be free of histamine and choline, and the active principle to be of protein-like nature.

Secretin.—Apparently no new progress has been made in recent years on the chemical structure of secretin. Comparative biological assays of crystalline secretin picrolonates, obtained in different laboratories, with a single reference standard have been carried out by Greengard & Stein (110). Incubation of secretin with blood serum destroys the activity of the principle; this is probably due to the presence in blood of an enzyme termed "secretinase" (111).

Pancreozymin.—Harper & Raper (112) prepared from the small intestine of the pig, dog, and cat a substance which on intravenous injection into cats causes an increased secretion of enzymes by the pancreas but has no effect on the volume of juice secreted; they named this substance "pancreozymin." The authors suggest that there may be a dual nervous and hormonal control of the secretion of enzymes by the pancreas and that pancreozymin is the hormone responsible for the latter.

Cholecystokinin.—A rapid and sensitive frog assay method has been worked out by Seager (113). The author also described briefly a modification of the method of Greengard & Ivy (114) for the preparation of cholecystokinin. Incubation of cholecystokinin with blood serum inactivates the hormone. The enzyme involved may or may not be secretinase (115).

Enterogastrone.—This principle, which inhibits gastric secretion, has been obtained in purified form from concentrates of the upper intestinal mucosa (116). Ivy and his associates (117, 118) have reported that administration of enterogastrone will prevent the development of gastrojejunal ulcer in the Mann-Williamson dog in 80 per cent of the cases.

Enterocrinin.—A method for assaying enterocrinin, the hormone

which stimulates the secretory glands of the intestines, has been described by Fink & Nasset (119). A detailed procedure for obtaining highly potent preparations of enterocrinin from the intestines has been described by Fink (120).

Urogastrone.—In the past few years evidence has been obtained for the existence of a substance in the urine which is probably an excretory product of enterogastrone, which has been named urogastrone, and which possesses a similar biological activity to that of enterogastrone. Methods for the preparation of extracts containing urogastrone from normal urine have been described. From its chemical behavior the active agent appears to be a complex organic base (121). The active principle has been shown to be distinct from pituitrin (122) and to possess characteristics which differentiate it from enterogastrone (123). Scott, Moe & Brunschwig (124) have presented evidence that the gastric secretory depressant factor is not identical with urogastrone.

Thymus

Whether the thymus is an endocrine organ is still undetermined. Reinhardt (125) reported that thymectomy by surgical methods has no apparent effect on the growth and development of the rat when performed at the age of one to two days and the effects studied past the onset of puberty. No evidence that the thymus or thymus extracts (thymocresin) exert an accelerating effect on growth and development of animals has as yet been obtained (126, 127, 128). The claim of Bomskow and his associates of the presence of a physiologically active, lipoid solvent soluble substance in thymus tissue has not been substantiated by other investigators (129 to 132).

Pineal

Fischer (133) described the preparation of a pineal gland fraction which inhibits the opening of the vaginal membrane in infantile female mice.

Antihormones

Many basic facts have yet to be established as to the specificity of the antihormones and to the antigenicity of hormones. The question whether chemically pure protein hormones are antigenic and the nature of the interaction between hormone and antihormone still has to be answered. Only the more recent literature on this subject will be dis-

cussed in this section. Several comprehensive reviews on antihormones have been published within the last three years (134 to 137).

Antigonadotrophic. — Katzman, Wade & Doisy (138) reported that the sera of sheep treated with sheep pituitary extract for as long as 262 days did not show antigonadotrophic activity. On the contrary, they continue to possess enhancing action on the gonadotrophic activity of this pituitary extract in immature female rats. The active factor of these sera also augments the action of rat pituitary extract but not that of pig and beef pituitaries or the urine of pregnant and castrated women. When administered alone, it produces no apparent effect on the reproductive tracts of virgin female rabbits and immature or mature female rats. Rowlands & Williams (139) found the progonadotrophic activity appearing in the serum of a goat injected with an extract of pig pituitary gland to be present in the globulin fraction. This globulin fraction, while augmenting the gonadotrophic activity of the same pig pituitary extract in intact rats, showed no progonadotrophic activity in hypophysectomized rats. Thompson & Melnick (140) observed that the inhibitory principle to human pituitary gonadotrophic hormone was contained in the gamma globulin fraction of goat antiserum. The gonadotrophic potency of the hypophyses of immature and adult female rats treated for sixty-three and fifty-four days, respectively, with antigonadotrophic serum was found by Meyer, Kupperman & Finerty (141) to be like that of castrated rats and greatly exceeded the gonad-stimulating power of the pituitary glands of normal rats. The gonads of the animals treated with antihormone were less than half the weight of those of the normal control females. The gonads of the adult female rats killed nineteen days after cessation of antihormone injection had entirely recovered from the atrophic state found immediately after discontinuing the antigonadotrophic treatment. The gonadotrophic content of the pituitary gland of these animals was the same as that of normal control rats. McShan, Wolfe & Meyer (142) reported that antigonadotrophic sera developed in rabbits and goats are more effective in preventing the gonad-stimulating action of relatively unpurified extracts prepared from sheep, hog, and cow pituitary glands when they and the gonadotrophin are injected separately into immature rats, than when mixed *in vitro* before injection. With the removal of much of the inert material from sheep pituitary extract it was found that combined injections of gonadotrophin and antigonadotrophic sera were as effective as when administered by separate injections. Marvin & Meyer (143) substanti-

ated the observations of Thompson (144) that the injection of pituitary gonadotrophic preparations results in the formation first, of a substance which augments the gonadotrophic action, and then of a substance which inhibits the effect of the gonadotrophin.

Kupperman, Meyer & McShan (145) observed that the injection of a purified follicle-stimulating hormone preparation produced an antiserum in rabbits. This antiserum showed no antiluteinizing effect but had a marked antifollicle-stimulating response. A crude pituitary extract injected into rabbits produced an antiserum which inactivated both luteinizing and follicle-stimulating hormones. Chow (146) prepared an antiserum by injecting a highly purified swine-luteinizing hormone preparation and found that precipitation will occur only with swine-luteinizing hormone preparation; sheep and beef-luteinizing hormone preparation failed to react. De Fremery & Scheygrond (147) observed the formation of antichorionic gonadotrophic activity in the blood and milk of goats which had been treated every other day for several months with 1,000 I.U. of a gonadotrophic extract from human pregnancy urine. Experiments of Zondek, Sulman & Sklow (148) show that chorionic gonadotrophic antiserum is 100 per cent effective against male and menopause urine gonadotrophin, but only 7 per cent effective against human hypophyseal gonadotrophin.

The injection of gonadotrophic preparations of equine origin elicits the production of antigonadotrophic activity in man (149, 150). Van den Ende (151) has shown that highly purified pregnant mare serum gonadotrophin in rabbits stimulates the formation of antibodies that may be precipitated by the antigen, and which induce an anaphylactic reaction *in vitro*. The precipitins in this case were found to be directed against constituents not related to the hormone. Gordon (152) reported that the more purified pregnant mare serum preparations evoke, in both rabbits and rats, a greater antihormone response than cruder samples. On the other hand Leathem & Abarbanel (153) found that increased purification of the equine gonadotrophin will markedly lower the tendency for the formation of antigonadotrophins in the human.

Antilactogenic.—Bischoff & Lyons (154) found mammotrophic preparations made from beef or sheep pituitaries to be antigenically indistinguishable. The crop-stimulating effect of beef and sheep mammotrophin was neutralized by injecting the pigeons with a sufficiently high dosage of antimammotrophic serum prepared in rabbits against either beef or sheep mammotrophin.

Antithyrotrophic.—Various investigators (155, 156) demonstrated

that administration of mammalian thyroglobulin stimulates the formation of physiologically active antihormones. Lerman (156) reported that in rabbit serum the antibodies to thyroglobulin are "organ specific" rather than "species specific." Reforzo-Membrivis (157) found that intraperitoneal injection into normal guinea pigs of a suspension of hypophyses of normal rats fed the usual diet produced the following deviations from normal: (*a*) a 35 per cent increase in the weight of the thyroid, which presented the typical histological picture of hyperfunction, (*b*) a 28 per cent increase in the oxidation index of the thyroid tissue, and (*c*) a 9 to 25 per cent increase in basal metabolism. Injection of hypophyses of normal or thyroidectomized rats fed desiccated thyroid produced: (*a*) a 26 per cent reduction in the weight of the thyroid and a 26 per cent decrease in the height of the thyroid epithelium, (*b*) a 26 to 48 per cent decrease in basal metabolism, and (*c*) a 23 per cent decrease of the oxidation index of the thyroid tissue. These results indicate that the hypophyses of rats fed thyroid develop a thyroid-inhibiting action which causes a decrease in the basal metabolism. The antithyroid effect may perhaps be due to the formation of paraxanthine (66).

Metabolism.—Ennor & Singer (158) found that the serum of a rabbit treated with an anterior pituitary extract abolishes the glycosuria in partially depancreatized rats and inhibits the diabetogenic action of pituitary extracts in the same animals. Such a preparation may perhaps be useful in the treatment of diabetes mellitus.

Insulin.—Lowell (159) has presented evidence for the existence of two antibodies for crystalline insulin in the serum of a patient who was both allergic and resistant to crystalline insulin.

LITERATURE CITED

1. Li, C. H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **146**, 627–31 (1942)
2. Schwenk, E., Fleischer, G. A., and Tolksdorf, S., *J. Biol. Chem.*, **147**, 535–40 (1943)
3. Fleischer, G. A., *J. Biol. Chem.*, **147**, 525–33 (1943)
4. Li, C. H., *J. Biol. Chem.*, **146**, 633–38 (1942)
5. Li, C. H., *J. Biol. Chem.*, **148**, 289–91 (1943)
6. Lahr, E. L., Bates, R. W., and Riddle, O., *Endocrinology*, **32**, 251–59 (1943)
7. Trentin, J. J., Lewis, A. A., Bergman, A. J., and Turner, C. W., *Endocrinology*, **33**, 67–74 (1943)
8. Lewis, A. A., and Turner, C. W., *Proc. Soc. Exptl. Biol. Med.*, **39**, 435–36 (1938)
9. Ciereszko, L. S., and White, A., *Federation Proc.*, **1**, 105 (1942)
10. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.*, **149**, 413–24 (1943)
11. Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, **149**, 425–36 (1943)
12. Neufeld, A. H., *Proc. Soc. Exptl. Biol. Med.*, **54**, 90–92 (1943)
13. Burtner, E. J., *J. Am. Chem. Soc.*, **65**, 1238 (1943)
14. Tyslowitz, R., *Science*, **98**, 225–26 (1943)
15. Simpson, M. E., Evans, H. M., and Li, C. H., *Endocrinology*, **33**, 261–68 (1943)
16. Marx, W., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **147**, 77–89 (1943)
17. Evans, H. M., Simpson, M. E., Marx, W., and Kibrik, E., *Endocrinology*, **32**, 13–16 (1943)
18. Marx, W., Simpson, M. E., Li, C. H., and Evans, H. M., *Endocrinology*, **33**, 102–5 (1943)
19. Marx, W., Simpson, M. E., and Evans, H. M., *Proc. Soc. Exptl. Biol. Med.*, **49**, 594–97 (1942)
20. Herring, V. V., and Evans, H. M., *Am. J. Physiol.*, **140**, 452–59 (1943)
21. Marx, W., Anderson, E., Fong, C. T. O., and Evans, H. M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 38–39 (1943)
22. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *Am. J. Physiol.*, **138**, 439–49 (1943)
23. Shipley, R. A., *Endocrinology*, **31**, 629–33 (1942)
24. Shipley, R. A., and Seymour, W. B., *Endocrinology*, **31**, 634–37 (1942)
25. Gray, C. H., *J. Endocrinol.*, **3**, 132–40 (1942)
26. Smith, R. B., Jr., *J. Pharmacol.*, **75**, 342–49 (1942)
27. Vaichulis, J. A., *Endocrinology*, **32**, 361–66 (1943)
28. Landgrebe, F. W., Reid, E., and Waring, H., *Quart. J. Exptl. Physiol.*, **32**, 121–41 (1943)
29. Abramowitz, A. A., Papandrea, D. N., and Hisaw, F. L., *J. Biol. Chem.*, **151**, 579–86 (1943)
30. Chen, G., and Geiling, E. M. K., *J. Pharmacol.*, **78**, 222–37 (1943)

31. VARIOUS AUTHORS, *Ann. New York Acad. Sci.*, **43**, 253–426 (1943)
32. MCSHAN, W. H., AND MEYER, R. K., *J. Biol. Chem.*, **151**, 259–66 (1943)
33. MEYER, R. K., MCSHAN, L. H., AND CASIDA, L. E., *Proc. Soc. Exptl. Biol. Med.*, **52**, 78–80 (1943)
34. CASIDA, L. E., MEYER, R. K., AND MCSHAN, W. H., *Am. J. Physiol.*, **139**, 89–94 (1943)
35. KATZMAN, P. A., GODFRID, M., CAIN, C. K., AND DOISY, E. A., *J. Biol. Chem.*, **148**, 501–7 (1943)
36. GURIN, S., BACHMAN, C., AND WILSON, D. W., *J. Biol. Chem.*, **133**, 467–76, 477–84 (1940)
37. BISCHOFF, F., *Endocrinology*, **32**, 260–62 (1943)
38. SALMON, A. A., AND HAMBLEN, E. C., *Endocrinology*, **33**, 257–59 (1943)
39. JONES, G. E. S., GEY, G. O., AND GEY, M. K., *Bull. Johns Hopkins Hosp.*, **72**, 26–38 (1943)
40. LEATHEM, J. H., AND MILLS, E. J., JR., *Endocrinology*, **31**, 318–22 (1942)
41. LEVIN, L., *Endocrinology*, **28**, 378–87 (1941)
42. EVANS, H. M., AND GORBMAN, A., *Proc. Soc. Exptl. Biol. Med.*, **49**, 674–78 (1942)
43. RIMINGTON, C., AND ROWLANDS, I. W., *Nature*, **152**, 355 (1943)
44. KUPPERMAN, H. S., MEYER, R. K., AND MCSHAN, W. H., *Endocrinology*, **29**, 525–30 (1941)
45. JENSEN, H., HAUSCHILDT, J. D., AND EVANS, J. S., *Proc. Soc. Exptl. Biol. Med.*, **50**, 356–58 (1942)
46. SMITH, P. E., *Endocrinology*, **31**, 1–12 (1942)
47. DAVIS, M. E., AND HELLBAUM, A. A., *J. Clin. Endocrinol.*, **3**, 517–25 (1943)
48. CEITHAML, J. J., AND KOCH, F. C., *Endocrinology*, **31**, 249–60 (1942)
49. BATES, R. W., AND SCHOOLEY, J. P., *Endocrinology*, **31**, 309–17 (1942)
50. HUF, E., *Z. physiol. Chem.*, **274**, 66–87 (1942)
51. BURDICK, H. O., WATSON, H., CIAMPA, V., AND CIAMPA, T., *Endocrinology*, **33**, 1–15 (1943)
52. BISCHOFF, F., *Endocrinology*, **30**, 667–70 (1942)
53. MORTON, M. E., CHAIKOFF, I. L., REINHARDT, W. O., AND ANDERSON, E., *J. Biol. Chem.*, **147**, 757–69 (1943)
54. MORTON, M. E., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **147**, 1–9 (1943)
55. SCHACHNER, H., FRANKLIN, A. L., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **151**, 191–99 (1943)
56. FRANKLIN, A. L., AND CHAIKOFF, I. L., *J. Biol. Chem.*, **148**, 719–20 (1943)
57. MACKENZIE, C. G., AND MACKENZIE, J. B., *Endocrinology*, **32**, 185–209 (1943)
58. ASTWOOD, E. B., SULLIVAN, J., BISSELL, A., AND TYSLOWITZ, R., *Endocrinology*, **32**, 210–25 (1943)
59. ASTWOOD, E. B., *J. Am. Med. Assoc.*, **122**, 78–81 (1943)
60. WILLIAMS, R. H., AND BISSELL, G. B., *Science*, **98**, 156–58 (1943)
61. REINEKE, E. P., AND TURNER, C. W., *J. Clin. Endocrinol.*, **3**, 1–6 (1943)
62. REINEKE, E. P., WILLIAMSON, M. B., AND TURNER, C. W., *J. Biol. Chem.*, **147**, 115–19 (1943)
63. LAUTENSCHLÄGER, C. L., AND BOCKMÜHL, M., *Z. physiol. Chem.*, **274**, 104–8 (1942)

64. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, **149**, 555–61 (1943)
65. Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, **149**, 563–70 (1943)
66. Carter, G. S., Mann, F. G., Harley-Mason, J., and Jenkins, G. N., *Nature*, **151**, 728–30 (1943)
67. Rawson, R. W., Sterne, G. D., and Aub, J. C., *Endocrinology*, **30**, 240–45 (1942)
68. Means, J. H., *Ann. Internal Med.*, **19**, 567–86 (1943)
69. Cutting, M., *Biochem. J.*, **36**, 376–83 (1943)
70. Lukens, F. D. W., and Dohan, F. C., *Endocrinology*, **30**, 175–202 (1942)
71. Lukens, F. D. W., Dohan, F. C., and Wolcott, M. W., *Endocrinology*, **32**, 475–87 (1943)
72. Reiner, L., Lang, E. H., Irvine, J. W., Jr., Peacock, W., and Evans, R. D., *J. Pharmacol.*, **78**, 352–57 (1943)
73. MacBride, D. M., and Roberts, H. K., *J. Am. Med. Assoc.*, **122**, 1225–31 (1943)
74. Colwell, A. R., and Izzo, J. L., *J. Am. Med. Assoc.*, **122**, 1231–36 (1943)
75. Hildebrand, A. G., and Rynearson, E. H., *Arch. Internal Med.*, **72**, 37–45 (1943)
76. Dunn, J. S., and McLetchie, N. G. B., *Lancet*, **245**, 384–87 (1943)
77. Bailey, C. C., and Bailey, O. T., *J. Am. Med. Assoc.*, **122**, 1165–66 (1943)
78. Goldner, M. G., and Gomori, G., *Endocrinology*, **33**, 297–308 (1943)
79. Reichstein, T., and Shoppee, C. W., *Vitamins and Hormones*, Vol. **I**, 345–413 (Academic Press, New York, 1943)
80. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 607–19 (1943)
81. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 705–15 (1943)
82. Lardon, A., and Reichstein, T., *Helv. Chim. Acta*, **26**, 747–55 (1943)
83. Shoppee, C. W., and Reichstein, T., *Helv., Chim. Acta.*, **26**, 1316–28 (1943)
84. Segaloff, A., and Nelson, W. O., *Endocrinology*, **31**, 592–96 (1942)
85. Kuizenga, M. H., Wick, A. N., Ingle, D. J., Nelson, J. W., and Cartland, G. F., *J. Biol. Chem.*, **147**, 561–65 (1943)
86. Reinecke, R. M., and Kendall, E. C., *Endocrinology*, **31**, 573–77 (1942)
87. Reinecke, R. M., and Kendall, E. C., *Endocrinology*, **32**, 505–8 (1943)
88. Bergman, H. C., and Klein, D., *Endocrinology*, **33**, 174–76 (1943)
89. Rogoff, J. M., *Penna. Med. J.*, **46**, 346–51 (1943)
90. Ingle, D. J., *Endocrinology*, **34**, 191–202 (1944)
91. Fraenkel-Conrat, H., Simpson, M. E., and Evans, H. M., *J. Biol. Chem.*, **147**, 99–108 (1943)
92. Fraenkel-Conrat, H., *Proc. Soc. Exptl. Biol. Med.*, **51**, 300–2 (1942)
93. Griffiths, M., *Proc. Linnean Soc. N.S. Wales*, **67**, 282–84 (1942)
94. Jensen, H., Grattan, J. F., and Hart, G. W., *Endocrinology*, **30**, 203–7 (1942)
95. Ingle, D. J., *Endocrinology*, **31**, 419–38 (1942)
96. Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, **148**, 455–56 (1943)
97. Shipley, R. A., Dorfman, R. I., and Horwitt, B. N., *Am. J. Physiol.*, **139**, 742–44 (1943)
98. Hoffman, M. M., Kazmin, V. E., and Browne, J. S. L., *J. Biol. Chem.*, **147**, 259–60 (1943)

99. Carnes, W. H., Osebold, J., and Stoerk, H. C., *Am. J. Physiol.*, **139**, 188–92 (1943)
100. Ross, W. F., and Wood, T. R., *J. Biol. Chem.*, **146**, 49–58 (1943)
101. Wood, T. R., and Ross, W. F., *J. Biol. Chem.*, **146**, 59–62 (1943)
102. Wood, T. R., and Ross, W. F., *J. Am. Chem. Soc.*, **64**, 2759–60 (1942)
103. Selye, H., *Arch. Path.*, **34**, 625–32 (1942)
104. Ingalls, T. H., Donaldson, G., and Albright, F., *J. Clin. Investigation*, **22**, 603–8 (1943)
105. Stoerk, H. C., *Proc. Soc. Exptl. Biol. Med.*, **54**, 50–53 (1943)
106. Ivy, A. C., *Glandular Physiology and Therapy*, 523–35 (Am. Med. Assoc., Chicago, 1942)
107. Komarov, S. A., *Am. J. Physiol.*, **126**, 558 (1939)
108. Komarov, S. A., *Rev. Can. Biol.*, **1**, 191–205 (1942)
109. Komarov, S. A., *Rev. Can. Biol.*, **1**, 377–401 (1942)
110. Greengard, H., and Stein, I. F., Jr., *Proc. Soc. Exptl. Biol. Med.*, **46**, 149–51 (1941)
111. Greengard, H., Stein, I. F., Jr., and Ivy, A. C., *Am. J. Physiol.*, **133**, 121–27 (1941)
112. Harper, A. A., and Raper, H. S., *J. Physiol.*, **102**, 115–25 (1943)
113. Seager, L. D., *Proc. Soc. Exptl. Biol. Med.*, **47**, 257–60 (1941)
114. Greengard, H., and Ivy, A. C., *Am. J. Physiol.*, **124**, 427–34 (1938)
115. Greengard, H., Stein, I. F., Jr., and Ivy, A. C., *Am. J. Physiol.*, **134**, 733–38 (1941)
116. Greengard, H., Grossman, M. I., Hands, A. P., and Ivy, A. C., *Federation Proc.*, **2**, 17–18 (1943)
117. Hands, A. P., Greengard, H., Preston, F. W., Fanley, G. B., and Ivy, A. C., *Endocrinology*, **30**, 905–11 (1942)
118. Hands, A. P., Greengard, H., Fanley, G. B., and Ivy, A. C., *Federation Proc.*, **2**, 18 (1943)
119. Fink, R. M., and Nasset, E. S., *Am. J. Physiol.*, **138**, 626–32 (1943)
120. Fink, R. M., *Am. J. Physiol.*, **139**, 633–37 (1943)
121. Gray, J. S., Wieczorowski, E., Wells, J. A., and Harris, S. C., *Endocrinology*, **30**, 129–34 (1942)
122. Gray, J. S., Harris, S. C., and Wieczorowski, E., *Proc. Soc. Exptl. Biol. Med.*, **46**, 691–93 (1941)
123. Harris, S. C., and Gray, J. S., *Federation Proc.*, **1**, 37 (1942)
124. Scott, V. B., Moe, R., and Brunschwig, A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 45–46 (1943)
125. Reinhardt, W. O., *Proc. Soc. Exptl. Biol. Med.*, **43**, 732–33 (1940)
126. Burrill, M. W., and Ivy, A. C., *Endocrinology*, **28**, 94–100 (1941)
127. Segaloff, A., and Nelson, W. O., *Endocrinology*, **27**, 693–99 (1940)
128. Reinhardt, W. O., Marx, W., and Evans, H. M., *Proc. Soc. Exptl. Biol. Med.*, **46**, 411–15 (1941)
129. Wells, B. B., Riddle, O., and Marvin, N. H., *Proc. Soc. Exptl. Biol. Med.*, **49**, 473–76 (1942)
130. Hoster, A. A., and Welch, J. B., *Proc. Soc. Exptl. Biol. Med.*, **51**, 201–3 (1942)
131. Albers, D., and Sasyk, Z., *Biochem. Z.*, **312**, 60–67 (1942)

132. Huf, E., and Ripke, O., *Arch. ges. Physiol.* (*Pflügers*), **245**, 802–18 (1942)
133. Fischer, E., *Endocrinology*, **33**, 116–17 (1943)
134. Collip, J. B., Selye, H., and Thompson, D. L., *Biol. Revs., Cambridge Phil. Soc.*, **15**, 1–34 (1940)
135. Thompson, K. W., *Physiol. Revs.*, **21**, 588–631 (1941)
136. Thompson, D. L., Collip, J. C., and Selye, H., *J. Am. Med. Assoc.*, **116**, 132–36 (1941)
137. Zondek, B., and Sulman, F., *The Antigonadotropic Factor* (Williams & Wilkins, Baltimore, 1942)
138. Katzman, P. A., Wade, N. J., and Doisy, E. A., *Endocrinology*, **25**, 554–67 (1939)
139. Rowlands, I. W., and Williams, P. C., *J. Endocrinol.*, **2**, 75–80 (1940)
140. Thompson, K. W., and Melnick, J. L., *Endocrinology*, **28**, 723–26 (1941)
141. Meyer, R. K., Kupperman, H. S., and Finerty, J. C., *Endocrinology*, **30**, 662–66 (1942)
142. McShan, W. H., Wolfe, H. R., and Meyer, R. K., *Endocrinology*, **33**, 269–75 (1943)
143. Marvin, H. N., and Meyer, R. K., *Endocrinology*, **32**, 271–78 (1943)
144. Thompson, K. W., *Proc. Soc. Exptl. Biol. Med.*, **35**, 640–44 (1937)
145. Kupperman, H. S., Meyer, R. K., and McShan, W. H., *Endocrinology*, **29**, 525–30 (1941)
146. Chow, B. F., *Endocrinology*, **30**, 657–61 (1942)
147. De Fremery, and Scheygrond, B., *J. Endocrinol.*, **2**, 357–61 (1941)
148. Zondek, B., Sulman, F., and Sklow, J., *Endocrinology*, **29**, 531–34 (1941)
149. Rowlands, I. W., and Spence, A. W., *Brit. Med. J.*, **2**, 947–50 (1939)
150. Jailer, J. W., and Leathem, J. H., *Proc. Soc. Exptl. Biol. Med.*, **45**, 506–8 (1940)
151. Van den Ende, M., *J. Endocrinol.*, **2**, 403–17 (1941)
152. Gordon, A. S., *Endocrinology*, **29**, 35–40 (1941)
153. Leathem, J. H., and Abarbanel, A. R., *J. Clin. Endocrinol.*, **3**, 206–11 (1943)
154. Bischoff, H. W., and Lyons, W. R., *Endocrinology*, **25**, 17–27 (1939)
155. Went, S., Piribauer, K., and Kesztyüs, L., *Arch. Exptl. Path. Pharmakol.*, **195**, 721–26 (1940)
156. Lerman, J., *Endocrinology*, **31**, 558–66 (1942)
157. Reforzo-Membrivis, J., *Endocrinology*, **32**, 263–70 (1943)
158. Ennor, A. H., and Singer, E., *J. Endocrinol.*, **3**, 118–21 (1942)
159. Lowell, F. C., *Proc. Soc. Exptl. Biol. Med.*, **50**, 167–72 (1942)

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THE WATER-SOLUBLE VITAMINS

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Introduction

In preparing a review of the developments of a year in a field as active as that of the water-soluble vitamins, one is confronted by an overwhelming literature representing the efforts of thousands of investigators from many laboratories. Unfortunately, some researches which might significantly add to our knowledge of this subject will probably not be discussed due to the unavailability of certain publications or to the oversight of the reviewer.

The discussion of the term vitamin, presented in the reviews of the previous years, will be omitted, since the year 1943 has not produced scientific developments to further clarify the present definition. However, those who have actively followed and participated in this field might approve the suggestion that before giving the name of a new vitamin to a substance believed to be present in crude fractions, its properties, both chemical and physiological, should be amply characterized.

The recognition of the occurrence of several active forms of some of the vitamins, for example, biotin, appears to have produced a need for a terminology which would indicate the relationship of these biologically active substances. The term "vitamer" has been suggested for this purpose (1). An objection has been made to this term based on the view that the wrong part of the word vitamin was used in the forming of vitamer. The terms isotel and isotelic were offered in its place (2). However, before accepting this terminology, it should be remembered that thus far very few substances except simple derivatives have been found among the water-soluble vitamins which serve the same purposes under all biological conditions. It is more often the case that one substance is able to replace another in specific biological reactions.

Thiamin

Assay methods and their application.—Even though the determination of this vitamin has received much attention in the past, certain modifications and improvements were reported this year. In a study in which the various animal (3 to 6) and chemical (7, 8)

assay methods were compared, the authors felt that the choice of any of the methods for a particular sample should be influenced by the potency of the material, the rapidity with which results are desired, and the sensitivity needed (9).

As the result of comparative tests conducted on an extensive scale, both a thiochrome (10) and an azo (11) method were found to be reliable for routine analyses of flour. Using the thiochrome method, the thiamin content of breads prepared from 80 per cent and 70 per cent extraction flour was investigated. The bread prepared from the former had twice as much thiamin as the latter (12). A rapid extraction method was applied to the estimation of thiamin in flour with the thiochrome value determined with a simple visual instrument. The results were found to be satisfactory (13). A procedure with this method was devised for determining the thiamin content in 5 cc. samples of oxalated whole blood, with results averaging 5.70 μg. per 100 cc. for normal human subjects, which agreed in general with those found by other methods (14). As a result of some modifications of the thiochrome assay (15), closer agreement with the bioassay values was obtained (16). In studying the stability of thiochrome in alkaline ferricyanide, hematin was found to catalyze its oxidative destruction (17). The thiochrome assay procedure has been applied to the determination of the thiamin content of wheat germ (18) and of various rice products (19).

A microbiological assay procedure, using as test organism *Streptococcus salivarius,* was described. Cocarboxylase was found to be about 40 per cent more active than the equivalent amount of thiamin by this method. However, the fact that the degradation products, the pyrimidine and thiazole portions, gave no response affords this method an advantage over other microbiological assay procedures for this vitamin (20).

The *Phycomyces* method of assay was applied to the determination of the distribution of thiamin in the potato plant (21) and in lake waters (22).

That pork muscle contains large quantities of thiamin is well recognized. However, when the intake of thiamin per pound of feed for pigs was raised from 1,318 to 3,447 μg., the thiamin content of the pork muscle was increased 100 per cent. The saturation point was reached with a ration containing approximately 5,800 μg. per pound. The pig is able by some unknown mechanism to accumulate unusually large amounts of thiamin in its tissues (23).

The effects of pH and the presence of electrolytes (24) and organic materials (25) on the stability of thiamin in solution were studied. It appeared that under the test conditions, the presence of certain salts, such as phosphates, increased the stability of the vitamin while the presence of some other salts, such as borates, had the opposite effect (24).

Enzymatic inactivation of thiamin.—The original suggestion (26) that the substance in fresh fish which destroys thiamin is an enzyme was confirmed by further investigations into the nature of this destruction. By treatment with acetone, a dry stable preparation was obtained (27). Various species of fish were analyzed for their content of this destructive enzyme. It appeared that all thirty-one varieties of fresh-water fish contained this active substance while all nine species of salt-water fish contained none. It was possible to prove that the tissues of living carp contained thiamin by prompt heat inactivation after sacrificing (28). In contrast to this observation was the finding that carp blood did not contain any demonstrable amounts of thiamin, and there were sufficient amounts of the enzyme present in the blood to destroy additional quantities of thiamin (29).

The nature of the enzymatic process which inactivates thiamin has been further characterized, and the products of the reaction have been identified. By the isolation of the two products 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-hydroxyethylthiazole from the reaction mixture, the reaction was demonstrated as one of hydrolysis. The complex nature of the process was shown by the different behavior of carp tissue and sodium chloride extracts of such tissue. The first stage involved the liberation of the thiazole moiety, which was effectively accomplished by either the tissue or extract. However, the liberation of the pyrimidine moiety, while proceeding rapidly with tissue, was found to proceed much more slowly with the salt extract of the tissue. It was observed with extracts that, whereas the thiazole portion of the thiamin molecule had been readily liberated, the pyrimidine portion was bound in the form of an intermediate. This was further acted upon by the tissue or more slowly by the extracts, which were found to contain varying amounts of this activity. On dialysis of the sodium chloride extract, only slight thiamin-destroying properties were found in either the dialyzable or non-dialyzable fractions. Restoration to full activity was obtained by the recombination of these two fractions (30).

The presence of some heat stable factor was believed to be re-

sponsible for the destruction of thiamin in cows' milk when incubated at 37°C. (31).

Pyrithiamin.—The name pyrithiamin (32) has been applied to the pyridine analogue of thiamin, 2-methyl-4-amino-5-pyrimidyl-methyl-(2-methyl-3-hydroxyethyl)-pyridinium bromide, first prepared in 1941 (33) and found to inhibit the growth of fungi (34). This compound was shown to have potent antithiamin activity. When it was administered to mice, a species which does not show the usual characteristic signs of thiamin deficiency (35), many of these signs were seen in all of the animals. It was found possible to prevent or cure this disease produced by the feeding of pyrithiamin by giving thiamin in an amount indicated by the ratio of one molecule of thiamin to forty molecules of the pyrithiamin fed (32). Thus we have an example of an analogue of a vitamin producing a deficiency condition in animals which is reversed by the vitamin. By using this compound as a tool, our knowledge of uncomplicated thiamin deficiency will be greatly enhanced.

Pyrithiamin was shown to bear the same relationship to thiamin as sulfapyridine does to *p*-aminobenzoic acid. The inhibitor-growth factor ratios were found to be comparable. However, it is quite probable that pyrithiamin would be of little value as a chemotherapeutic agent since it was observed that non-toxic levels of this compound were not antibacterial in the blood of mice (36).

Human requirements.—The problem of establishing the minimal daily requirements of man has been further studied. With subjects on various levels of thiamin in a ten to twelve weeks' period, it was found that on an intake of 0.23 mg. per one thousand calories (on a three thousand-calorie ration) performance was as good as with an intake of 0.63 mg. per one thousand calories (37). However, the excretion values at this low level did not reach equilibrium until the fifth week, indicating the utilization of the vitamin stored as a result of large intake in the pre-experimental period. Unfortunately, due to the lack of negative control values on the performance tests, it is impossible to be certain that these tests would be affected by deficiency of the vitamin except of course in the condition of clinically recognizable deficiency.

In another study in which the effect of restricting the average daily intake to a total of 0.35 mg. (0.175 mg. per one thousand calories) was investigated over a period of 120 days, symptoms and signs of thiamin deficiency were observed as early as the thirtieth day. These signs

and symptoms progressed from decreased excretion of the vitamin followed by anorexia and weakness to definite polyneuropathy at about the 110th day. The progression of the deficiency was accompanied by higher blood pyruvic acid curves after the administration of glucose (38, 39).

Using as a criterion the absence of any thiamin in urine voided under specific fasting conditions, an average intake of 0.47 mg. a day was found to satisfy these minimal requirements. It was claimed that the presence of any vitamin in the urine collected under the test conditions indicated a surplus in the body, and that, therefore, no deficiency of the vitamin could exist under these circumstances. In subjects maintained at this level of vitamin intake, 0.47 mg. a day for four months, no clinical evidence of thiamin deficiency was observed (40). Further confirmation of this method of determining the level of thiamin nutrition is necessary before proper evaluation of the significance of this work is possible. Details of the assay method used for the determination of these very small quantities of thiamin have not been published as yet.

The possible effect of intestinal synthesis on dietary thiamin requirements has been discussed under the subject of the sulfonamides (41).

Miscellaneous observations.—The method of needle biopsy of muscle tissue (42) has been applied to the study of the thiamin content of human tissues. By use of this procedure, it was found that the content in muscle was higher in infants than in adults, whereas concentrations of thiamin in cerebral tissue increased with age (43). The effect of thiamin on basal metabolism has been studied (44). The metabolic rate in normal subjects was found to be increased to a much lesser degree when the thiamin intake was restricted during the thyroid hormone administration (45). Thyrotoxic subjects were observed to have lowered levels of blood thiamin and diphosphothiamin. A tendency of such patients to excrete more thiamin than normal subjects was also noted (46). It may well be that products of nuclear disintegration and other substances which are formed as a result of irradiation with roentgen rays require thiamin in more than normal amounts for their metabolism. Administration of thiamin was reported to be effective in preventing the symptoms of roentgen sickness (47).

Thiamin was reported to be slightly more effective than vitamin A and much more so than riboflavin in producing improvement of color vision (48).

When 20 mg. of thiamin were given by injection to two cows suffering from ketosis, there was some decrease in blood ketones and increase in blood sugar. While suggestive, the results were not conclusive (49). Small doses of thiamin were reported to have proved of value in the treatment of many cases of hyperinsulinism recognized clinically as hypoglycemia (50, 51). In anesthetized dogs the resistance to shock induced by hemorrhage was found to be dependent on the plasma thiamin level. With the higher levels much more severe bleeding was required before severe hypotension developed (52). Additional claims for the activity of thiamin in "fright" disease in dogs were reported. The author states that the vitamin must be injected, frequently for several doses, to be effective (53).

No beneficial effects were obtained by thiamin therapy in diabetic neuritis (54), advanced polyneuritis of pregnancy (55), or toxemia of pregnancy (56). Normal children and those having paralytic poliomyelitis showed no significant differences in excretion of thiamin (57). Thiamin deficiency did not increase the susceptibility of rats to Flexner's M. V. poliomyelitis cotton rat-adapted strain (58). Thiamin in large doses had no significant synergistic effect on the laxative action of phenolphthalein in the monkey (59). The factors concerned in determining the thiamin content of breast milk were investigated. The effectiveness with which dietary thiamin could influence the thiamin content of the milk was found to vary among women (60). Psychic disturbances have been suggested to be among the earliest manifestations of thiamin deficiency in man (61). The diagnosis and treatment of thiamin deficiency have been reviewed (62).

RIBOFLAVIN

Microbiological and fluorimetric methods of assay were studied collaboratively (63) with comparable results for the two methods. Whole wheat flour, for example, was found to contain 1.15 μg. of riboflavin per gram by microbiological methods and 1.11 μg. per gram by fluorimetric methods. It was observed that certain pigments occurring in bread may interfere with the fluorimetric assay (63).

Physiology.—The role of the various portions of the intestinal tract (64) of the rat in the absorption and excretion of riboflavin was investigated. The results showed that following bilateral nephrectomy, riboflavin administered intravenously was rapidly excreted into the small intestine. Destruction of the vitamin occurred quickly in the large intestine.

In comparing the effect of inanition (65) and that of riboflavin deficiency on the blood picture of the rat, it appeared that the changes in the number and distribution of the leucocytes probably resulted from the inanition associated with this deficiency.

The observation that the administration of thiamin (66) caused an increase in the riboflavin excretion, suggested the possibility that the extensive feeding of thiamin would produce riboflavin deficiency. However, even though large amounts of thiamin were given for as long as seventy-three days, no clinical or chemical signs of riboflavin deficiency were observed.

Biological synthesis.—The difference in the form in which corn was fed to steers (67) influenced the amounts of riboflavin synthesized in the rumen. Ground corn gave increased amounts above that obtained with whole corn. In general, however, the synthesis of riboflavin in the rumen was related to the carbohydrate in the ration.

Clinical studies and animal experiments.—The total riboflavin and riboflavin adenine dinucleotide in ox ocular tissue (68) was found to be maximal in the lacrymal and meibomian glands, suggesting that the corneal epithelium may get some of its flavin from the eye secretions (68). Vascularization of the cornea and symptoms of eye fatigue were reported to be quite common in healthy young adults and were associated with inadequate intake of riboflavin. Administration of 9.9 mg. daily resulted in a progressive decrease in the vascularity (69). However, not all cases of ariboflavinosis, as characterized by lesions of lips, tongue, or cornea, responded to riboflavin therapy (70). It may well be that the period of dosing in these cases was too short for the complete evaluation of the efficacy of the vitamin in the treatment of these signs. In some of the cases of so-called "rosacea keratitis" (71) there may be failure to obtain satisfactory responses with oral administration, probably due to faulty absorption from the intestinal tract. In such instances, parenteral injection of 1 mg. doses was found effective (71). Certain signs of deficiency, such as lesions of the mouth (72), should not be confused with those due to perlèche, which may be caused by such conditions as dental defects and dribbling of saliva. These latter lesions, of course, will not respond to riboflavin therapy (72).

Ariboflavinosis and mild symptoms of pellagra have been observed following gastric surgery (73). Riboflavin appeared to be of value in the treatment of decubital ulcerations (74).

Addition of riboflavin to diets already containing adequate

amounts did not increase the growth rate of spontaneous tumors (75) in mice, as contrasted with the decrease of the growth rate on deficient diets. There was, however, an increase in the number of spontaneous tumors in the tumor-bearing animals fed the high level of riboflavin (75).

In experiments with rats, the significance of a liberal level (76) of riboflavin, 10 µg. per gram of diet, over a level of 3 µg. per gram which supported adult vitality, was found in the more favorable growth and greater ability to withstand deprivation of the vitamin in the offspring of the respective families (76). From the evidence obtained by studies with human subjects (77), an intake of 0.5 mg. per thousand calories was found to be sufficient for maintaining satisfactory tissue levels.

Nicotinic Acid

Assay methods and their application.—Various improvements and modifications in both the colorimetric and microbiological methods used for the determination of nicotinic acid and its amide continue to appear in the literature. A procedure by which colorless cereal extracts are prepared by adsorption and elution from Lloyds' reagent has been devised (78). An increase in the specificity of the cyanogen bromide and aromatic amine test (König's reaction) for nicotinic acid in blood samples was accomplished by the pretreatment of the blood sample with a specific enzymatic system of microbial origin (79).

Some attention has been given to the problem of differentiating nicotinic acid from its amide in analytical procedures, a problem which chiefly concerns pharmaceutical chemists. By use of the time reaction, measurements of the color development by the König reaction gave results which were reproducible in the determination of nicotinic acid and nicotinamide in mixtures (80, 81). By converting nicotinamide in mixtures to the biologically inert β-aminopyridine by treatment with bromine and potassium hydroxide (Hoffman's reaction), which does not affect nicotinic acid, the usual microbiological methods for nicotinic acid can be applied. Thus, the two biologically-active forms can be differentiated by assays before and after treatment (82).

Improvements in the microbiological assay for nicotinic acid with the *Lactobacillus arabinosus* were made by use of a new medium which gave an enhanced response to nicotinic acid (83). A close agreement was found between the microbiological and chemical

methods (König's reaction) in the assay of various pharmaceutical B complex products (84).

The production of a growth factor which will replace or substitute for nicotinamide for several strains of *B. dysenteriae, Staphylococci,* and *L. arabinosus* was accomplished by the heating at 100°C. of a neutral solution of glutamic acid and asparagine. The nature of the product is unknown (85).

Biochemistry.—The fluorescent material, F_2 (86), which normally appears in human urine in small amounts and in larger amounts after the administration of nicotinic acid or its amide, has been now shown to be N′-methylnicotinamide (87). It was isolated in crystalline form from urine and compared and found identical with the synthetic compound (87). Since the usual method for the determination of trigonelline (88) would also include this compound, an assay method (89) was devised. By use of this method, it was found that when nicotinamide is incubated with rat liver slices, it is converted aerobically to its N′-methyl derivative (90). This does not occur with nicotinic acid. Kidney or muscle does not methylate nicotinamide *in vitro.* The liver is, therefore, the probable site for this biological methylation (90).

A difference was shown between the lactose fermenting and non-lactose fermenting yeast in their ability to synthesize nicotinic acid. While the former required an external source of this vitamin, the latter was able to synthesize sufficient amounts for optimum growth (91).

The influence of nicotinic acid and thiamin on the glucose metabolism of *Staphylococcus aureus* was studied. It was observed that while the presence of nicotinic acid was essential, the effect of thiamin was to increase 2½ fold the amount of glucose metabolized by its activity in catalyzing the oxidation of pyruvic acid (92).

Animal requirements.—The requirement of the chick for nicotinic acid is only partly satisfied (about one-sixth) by its own synthesis (93). Chicks on a low nicotinic acid diet were reported as showing poor growth with dermatitis on the upper part of the feet and on the legs. Poor feathering was also observed (93). The minimal daily requirement of young growing swine (94) was found to be between 5 and 10 mg. per hundred pounds of pig.

Increasing the size of a single dose of nicotinic acid increased the period of protection from blacktongue in dogs, not proportionally, but logarithmically with the size of the dose (95). This indicates only

a definite limited storage of this vitamin in the dog, which is in agreement with the results obtained with rats (96).

Corn as a factor in pellagra.—That corn meal itself may be an etiological factor in pellagra was suggested by the observations made with dogs maintained on corn meal-containing Goldberger diets (97). Dehydration, lack of thirst, and hemoconcentration characteristic of both human pellagra and blacktongue produced with corn meal diets did not arise from simple nicotinic acid deficiency. Dogs on a purified ration in which the nicotinic acid content was very low did not show evidence of dehydration or lack of thirst (97) observed with corn meal-containing diets.

Clinical and other observations.—In a carefully controlled series of experiments with active young men, it was found that the efficiency with which a severe test requiring great physical exertion and coordination was performed could be increased by the addition of nicotinamide to their diet (98).

The administration of nicotinic acid to diabetics resulted in improved carbohydrate tolerance (99). The use of nicotinamide in conjunction with the barbiturate-hyoscine method of obstetric analgesia was found advantageous: there was less restlessness in the mothers, and fewer than usual babies required resuscitation (100). What may have been considered an undesirable property of nicotinic acid in affecting the vascular system has found valuable application as an adjuvant in the treatment of asthma (101). It also has been found satisfactory for the relief of vasospasm (102).

Pellagra has been reported to have developed among chronic psychotic (103) patients, probably resulting from habits of feeding, and Flexner's dysentery (103).

Adequate amounts of nicotinic acid and vitamin A were found to be essential factors in the control of calf scours (104).

Pyridoxine

Methods for the determination of pyridoxine still receive considerable attention. An assay method using the growth of rats gave dose response curves which were linear in the range of from 1 to 8 µg. (105). This and other biological assay methods have suffered in the past from the same drawbacks, namely the possible influence of any unknown or unrecognized growth stimulatory factors which may exist in foodstuffs. However, since the knowledge of the vitamins has advanced considerably in recent years, we can now apply biological assays with

some certainty that the influence of unknown factors will not be serious.

A method using a yeast strain which responds to pyridoxine (106), and another using an x-ray-induced mutant of the mold *Neurospora sitophila* have been described. Accuracy and specificity were indicated for the latter since the results were in good agreement with values obtained by animal assay (107). Using a modification (108) of the yeast assay (109) which was found to overcome some of the inadequacies of the original method, various biological materials were assayed for bound and free pyridoxine. By autoclaving the test preparation, either as a suspension or a solution, for thirty minutes at fifteen pounds pressure in 2 *N* sulfuric acid, bound pyridoxine was liberated in the free form which was then readily utilized by the growing yeast. It was observed that most of the naturally occurring pyridoxine is in the bound form (108).

Modification of the colorimetric assay was effected chiefly through the changes in the preparation of the sample prior to the coupling with diazotized sulfanilic acid (110).

Alanine was found to replace pyridoxine effectively for the growth of *S. lactis* indicating the possible synthesis of this vitamin from alanine. Structural relationships also show that this amino acid may be the biological precursor of the vitamin (111). It was also shown that the x-ray mutant of *N. sitophila,* the organism used in the microbiological assay mentioned above, was able to synthesize pyridoxine under specific conditions of pH and nitrogen nutrition (112).

The yellow compound present in the urine of pyridoxine-deficient rats (113) was isolated and identified as xanthurenic acid, 4,8-dihydroxy-quinoline-2-carboxylic acid, and was shown to arise from the tryptophane in the diet. The administration of pyridoxine or the removal of tryptophane from the diet caused the disappearance of xanthurenic acid (114) from the urine offering further evidence for the correlation of pyridoxine with protein metabolism (115).

Another synthesis of pyridoxine has been described (116). The original observation made in 1935 (117) that the vitamin is destroyed by light was confirmed by using physical, chemical, and microbiological methods of assay for measurement of the losses incurred at various pH values (118). Rapid destruction of pyridoxine by light was found to take place in neutral or alkaline solutions though there was very little loss at pH 1 (118).

Pyridoxine deficiency signs in turkeys were described as loss of

appetite, poor growth, apathy, hyperexcitability when disturbed, and convulsions (119). This vitamin was also found necessary for egg production by pullets (120). The anemia, epileptiform convulsions, and fatty infiltration of the liver of swine resulting from the deficiency of this vitamin (121, 122) were further investigated (123). In mice, pyridoxine appeared to affect the rate of growth of Sarcoma 180 (124). Pyridoxine was found useful as an adjunct to other therapy in the treatment of seborrheic or seborrhoid eruptions in humans (125).

The significance of pseudopyridoxine in the nutrition of rats (126) and some molds (127) has been questioned. It appears that this substance did not have the enhanced activity which has been found for it with lactic acid organisms, such as *S. lactis* (126, 128). However, in neither of these reports was sufficient experimental evidence given to justify the statement "that the physiological activity is limited to the lactic acid bacteria" (126).

Since pyridoxine has been shown to be essential for the synthesis of hemoglobin by certain species, its possible relationship to another iron-porphyrin complex, catalase, was investigated. It was shown that deficiency of pyridoxine did not significantly affect the catalase activity of the liver, kidney, and heart tissue of rats (129).

Pantothenic Acid

Assay methods.—The difficulties of reproducing by microbial methods the pantothenic acid values found by the chick assay have not as yet been overcome. Modifications of the basal medium for the test organism, *L. casei,* have been reported. Changes in the method of preparation of the samples were also tried (130). The presence of a combined form of pantothenic acid (131) in liver may account for part or all of the disagreement between the two assay methods. This combined form, which is stable to alkaline hydrolysis, is utilized by the chick but not by the test microorganism, *L. casei.* Enzymatic treatment converts it to the alkali labile form which is then available as pantothenic acid to the test organism (131). How the labile linkage between the lactone and β-alanine moieties in the pantothenic acid molecule in the combined form is protected from the hydrolytic action of alkali awaits further elucidation. In another report the presence of two forms of pantothenic acid in blood was indicated, one free, the other combined with protein (132).

Other improvements in the assay medium such as increasing the carbohydrate and nitrogen source, asparagine, were suggested and greater growth with certain preparations resulted, indicating the possible presence of stimulating factors (133). However, other workers suggested that the high titration values thus obtained were explainable on the basis that these extracts contained appreciable carbohydrate and substances capable of acting as buffers. They were able to demonstrate that the simple addition of glucose and sodium acetate would also give rise to higher acid production (134).

By the use of the changes in optical rotation, the rate of destruction of calcium pantothenate by hydrolysis was studied (135). The rate of hydrolysis while not only a function of temperature and pH is also affected by the presence of other substances (135).

A method for the assay of β-alanine using the growth of diphtheria bacilli has been developed (136). By this method, various tissues and proteins were investigated and the presence of β-alanine was revealed in such materials (137) which had previously been reported as not containing this compound (138).

Effect of compounds related to pantothenic acid.—The production of signs similar to those of pantothenic acid deficiency was obtained by dosing mice with pantoyltaurine (139). The correction of this condition by the administration of additional quantities of pantothenic acid was not reported. However, another report has appeared in which this compound, pantoyltaurine, also called thiopanate, was tested exhaustively without any pantothenic acid deficiency being observed in the test mice (140), which observation thus brings into question the significance of the previous communication. The inefficacy of pantoyltaurine in producing pantothenic acid deficiency was confirmed in studies with rats (141).

α-Methyl pantothenic acid was prepared by the condensation of α-methyl-β-alanine with the pantothenic acid lactone moiety. Its growth stimulating and inhibiting properties were investigated (142). However, the compound was quite impure as indicated by its elementary analysis so that no definite activity can with certainty be ascribed to this compound from the report.

Miscellaneous observations.—The requirements of hens in order to maintain maximum reproduction on a heated ration was found to be from 1,200 to 1,700 μg. of pantothenic acid per 100 gm. of ration. A diet containing only 200 μg. of pantothenic acid per 100 gm. of heated ration was, however, satisfactory for weight maintenance and liva-

bility (143). The influence of other heat labile factors destroyed in the preparation of this ration is difficult to determine.

Confirmation of the previously postulated role of pantothenate (144) in the oxidation system of pyruvic acid in washed suspensions of *Proteus morganii* was obtained by another worker (145). Thus, we have the addition of another vitamin as a component of pyruvate oxidase.

Excess pantothenic acid did not affect the rate of intestinal absorption of galactose. However, in deficient rats the rate was reduced about 15 per cent below that of the controls (146).

Some increase, about 24 per cent, in the average size of litters of mice and rats was found to result from the supplementation of a stock ration with substantial quantities of pantothenic acid (147). No influence on the rate of tumor growth was observed in deficient mice (124).

The effect of the deficiency of this vitamin on hair pigmentation has received much attention. In a study of black rats on a deficient diet, the loss of color could be accounted for by the atrophy of the hair bulbs and follicles and cessation of melanin deposition (148). The administration of calcium pantothenate has been found to produce no significant change in the graying of human subjects (74, 149).

Choline

An improvement in the method for the colorimetric determination of choline was achieved by a development of the usual reineckate procedure, making use of the colorimetric measurement of the chromium in the precipitated reineckate. With this modification as little as 15 µg. could be determined in samples (150).

The choline content of various products and feeds has been investigated. In general, animal products were found to be among the best sources (151, 152).

The requirement of turkey poults for the prevention of perosis due to choline deficiency was between the levels of 0.18 and 0.25 per cent of the ration (153). Other factors were found to influence the incidence of perosis (154). Preliminary observations were made which showed that there were differences in the choline requirement of different strains of rats (155).

Thiamin fatty livers were found to respond to choline, while this vitamin was found to be only partially lipotropic for cholesterol fatty livers and only slightly for biotin fatty livers (156). By making use of the experimental procedure which would allow the liver of a rat to

grow while the rat itself was losing weight as a result of partial hepatectomy and the feeding of large quantities of nicotinamide, it was found that neither choline deficiency nor a mixed thiamin-choline deficiency prevented the rapid regeneration of the liver. From the result of these experiments, it was felt that fatty livers of choline deficiency would only develop when the diet deficient in choline allowed moderate growth in young rats (157). The repression of growth effected by mineral deficiency had the same effect as that of the vitamins in preventing fatty livers in choline deficiency (158).

The lipotropic effect of inositol in the presence of other members of the B complex was found to be less than that produced by feeding both inositol and choline (159). In human patients with cancer of the gastrointestinal tract, inositol appeared to account for the lipotropic properties of lipocaic (160).

Inositol

The phospholipid containing inositol (161, 162) has been isolated (163) from soy bean phosphatides. The method used was described (163). By analysis, this compound was found to contain 16 per cent inositol, 15.5 per cent galactose, 8.3 per cent *d*-tartaric acid, 23.6 per cent fatty acid as oleic, an equivalent amount of unsaturated fatty acids, phosphoric acid, and ethanolamine. The name lipositol has been proposed for this compound (163).

Inositol has been reported as having possible value in the treatment of some skin diseases (74). Oral administration of one or two grams had no apparent ill effect (74). Patients with psoriasis have been treated with wheat germ or soy bean lecithin with some benefit (164).

The inhibition of tumor growth in mice transplanted with Sarcoma 180 was effected by the intravenous injection of inositol (165). The degree of inhibition was found to be dependent on the size of the dose injected.

Biotin

Chemistry.—The developments in the chemistry and biochemistry of biotin covering the period just previous to that to be discussed here have been thoroughly summarized and reviewed (166). The interesting chemistry which led to the elucidation of the chemical structure of 2′ keto-3,4-imidazolido-2-tetrahydrothiophenevaleric acid (167) is described (166). This chemical structure was demonstrated to be correct

by its synthesis (168). The comparison of the physical, biological, and microbiological properties proved that the synthetic product was identical with that isolated from natural sources (168).

Biologically active forms of biotin.—The biotin activity as measured by yeast growth (169) was determined for desthiobiotin, the product obtained by the action of Raney nickel on biotin (167). With this assay method, this non-sulfur-containing compound gave the same stimulation as biotin on a weight basis (170). On the other hand, it was found inactive for *L. casei*. It was also combinable with avidin (170), a property which has been attributed to the presence of the intact urea grouping (171). It will be of interest to learn whether the yeast in utilizing this degradation product reverses the action of the Raney nickel and reintroduces the sulfur atom into the molecule.

By use of several of the properties of biotin, such as its formation of a stable product with avidin and the stimulation of either yeast or *Rhizobium* growth, the existence of various biotin-like substances (vitamers) was indicated (1). Miotin, the heat-labile avidin-uncombinable fraction in urine while effectively replacing biotin as a growth factor for yeast, is inactive for *Rhizobium*, another biotin-requiring organism. The residual heat-stable avidin-uncombinable fraction resulting from the autoclaving of miotin was assigned the name tiotin. Another, a third form of biotin, which replaces biotin as a growth factor for *Rhizobium*, but is not active for yeast, was assigned the name rhiotin. It was avidin-combinable and stable to autoclaving. From chemical studies, not reported, it was claimed that these three substances are closely related chemically to biotin (1).

A study was made of the carbon dioxide pressures required for the growth of yeast with biotin and also the diamino carboxylic acid degradation product resulting from the removal of the carbon monoxide group of the urea ring of biotin (1). It was observed that with the latter a higher carbon dioxide pressure was required for growth, indicating its conversion to biotin by the growing yeast (1). When yeast is grown with an avidin-uncombinable form of biotin, such as tiotin, this form is converted into the avidin-combinable form. It was suggested that the alternation between the avidin-combinable and avidin-uncombinable forms may act in a system for the transfer of carbon dioxide as coenzyme (1). While this is an interesting hypothesis, further experimental evidence is required for proper evaluation.

Contrary to the reported inactivity of the methyl ester of biotin for

L. casei (172), it was found that this organism can utilize this derivative of biotin, but at a somewhat slower rate (173).

Some question has been raised as to whether the biotin crystals isolated from egg yolk and liver were identical. The former, α biotin, was reported to have a rotation of $[\alpha]_D^{21} = +51°$, and the latter, β biotin, $[\alpha]_D^{21} = +91°$ (174). However, both forms have the same empirical analysis, and approximately the same biological activity.

Assay methods.—An improvement in the medium used in the yeast assay for biotin was effected by the addition of amino acids in the form of biotin-free casein hydrolyzate (175). Factors producing nonspecific stimulation were thus eliminated. The range of greatest practicability of the test was reported to be from $1 \times 10^{-4\mu g.}$ to $3 \times 10^{-4\mu g.}$ (175).

The procedure of liberating biotin from samples of meat by autoclaving for two hours in 6 *N* sulfuric acid followed by assay with *L. casei* gave results which agreed with those already reported (176).

Several improvements in the *L. casei* (172) assay for biotin were made by changes in the norite eluate supplement and the inoculum (177).

Avidin-biotin.—In studying the possible interrelationships between bound biotin (AB, avidin-biotin) and biotin, it was found that while proteolytic enzymes of the body such as pepsin, trypsin, or pancreatin, or even incubation with tissues such as liver, kidney, muscle, or blood, could not liberate biotin from AB, oxidative procedures such as treatment with 0.45 per cent hydrogen peroxide were effective. As much as 10 to 20 per cent of the bound biotin was liberated by this treatment in a yeast-active form. It is therefore likely that a possible attack on AB in the body may be through some oxidation-reduction process (178) which would explain the effectiveness of AB when administered parenterally to rats (179).

From quantitative assays for avidin made on the various portions of the oviducts of laying and non-laying hens, it appears that the production of avidin is associated, either directly or indirectly, with the reproductive function of the ovary (180). The evidence that the administration of stilbestrol followed by progesterone induced the production of avidin in the oviducts of immature chicks and non-laying hens bears out this observation (181).

The use of avidin to decrease the free biotin intake of two subjects with cancer was reported. It was believed that by the elimination of an essential factor from the diet, such as biotin, growth of malignant

tissue could be checked (182). This experiment seemed especially worth while in that several types of carcinoma have been reported to contain abnormally high concentrations of biotin (183). Even though large quantities of avidin in the form of egg white were fed, no clinical signs of biotin deficiency were observed. Neither was the clinical course of these patients affected (182). However, there is one comment that may be made here, namely, that in these cases the avidin was administered essentially separately from the rest of the diet whereas in rats in the usual methods used for the production of biotin deficiency, the avidin has been mixed with substantially the entire diet. The removal of biotin as AB may not occur as efficiently in the body as elsewhere. It is interesting to note that in a previous report on the production of biotin deficiency in man the avidin was incorporated into the diet (184).

Biotin deficiency and disease.—Interesting possibilities for the role of biotin in certain infections were suggested by the observations that the period of infection with *T. lewisi* in biotin-deficient rats was longer than that in rats not deficient in biotin, and the administration of biotin during the infection shortened the period in deficient rats (185). However, it may well be that this effect is not entirely specific for biotin deficiency, since the effect of other vitamin deficiencies was not studied. Of especial current interest was the report that the level of biotin in chicks and ducks greatly influenced the severity of malarial infection (186). Among the biotin-deficient animals, the parasite number remained at a high level longer and there was a higher mortality than among the controls. An unusually high level of biotin in the plasma and red blood cells during the height of the infection, which returned to normal eight days after inoculation, may be related to the elimination of the parasites from the blood (186). Presumably this is another instance of a vitamin deficiency playing an important role in susceptibility to an infectious disease.

Other experiments with biotin.—In studies with the golden hamster, biotin was found necessary for good growth and reproduction (187). Although producing no growth stimulation in monkeys, biotin was found to be needed for maintaining the normal condition of the skin and coat of these animals (188).

In studying the effects of biotin deficiency on the capacity of the motor nerve to elicit tension in its muscles, no impairment was found (189). The rates of regeneration following denervation were also not affected by biotin deficiency (189).

The role of biotin in rats fed sulfonamide-containing diets has been discussed in the section of this review dealing with the effect of the sulfonamides.

THE NORITE ELUATE FACTOR ("L. CASEI" FACTOR), FOLIC ACID, VITAMIN B_C, AND THE "STREPTOCOCCUS LACTIS R" FACTOR

The terms norite eluate factor (*L. casei* factor) and folic acid have been used interchangeably following the observation that concentrates of the norite eluate factor (*L. casei* factor) were effective for the test organism, *Streptococcus lactis* R, which is used for the measurement of folic acid. Folic acid concentrates were also found to be effective in the assay for the norite eluate factor using the test organism *L. casei*.

The term vitamin B_c was originally applied to the factor in extracts of liver which was required by the chick for the prevention of an anemia of the hyperchromic macrocytic type when fed a purified diet (190, 191). The addition of sulfaguanidine to less purified diets was shown also to produce this anemia in chicks (192).

Vitamin B_c has now been obtained from liver in crystalline form both as the free acid and its methyl ester. It was obtained after repeated recrystallizations in clusters of thin yellow spear-head shaped crystals which did not melt below 360° C. with darkening beginning at about 250° C. Elementary analysis gave the following composition: C, 50.50, 50.63; H, 4.78, 4.78; N, 19.91 (193). The isolated material was found to be acidic in nature, forming salts with bases and esters with alcohol. It was found to be insoluble in the common organic solvents. While acids (pH 1) readily destroyed the activity of the compound, the destruction in alkali was much slower (192). The addition of 2.5 µg. per gram to the deficient diet resulted in normal growth with no anemia in chicks. It was also reported as highly active for *L. casei*, a concentration of 0.00005 µg. per cc. of medium producing half maximal growth. Because of this activity for *L. casei*, it appeared that this factor, the norite eluate factor, and folic acid were the same (193).

The isolation of two substances, highly active for *L. casei* from liver and yeast, was also reported (194). The product from liver had the same activity for *L. casei*, and the analysis of the methyl ester when calculated to the free acid form was the same as obtained with crystalline B_c. It is interesting to note that whereas the product isolated from yeast had about the same activity for *L. casei* as the product

from liver, its activity with *Streptococcus lactis* R was approximately one-half that of the liver preparation.

There was also reported the isolation of a compound, named tentatively *Streptococcus lactis* R (SLR) factor (195), which effectively replaced a folic acid preparation for *Streptococcus lactis* R but was ineffective for *L. casei* (196). However, it was shown that the organism *S. lactis* R converted the SLR factor into a form which was active for *L. casei* (195).

Thus we are in a quandary as to what is meant by folic acid. Is all activity as measured by the *Streptococcus lactis* assay folic acid? Then the isolated material, the SLR factor, which has been found to be active by this assay, should be folic acid. But this substance has been found inactive for *L. casei,* whereas folic acid concentrates were active for this organism. Furthermore, the activity of the two crystalline substances, from yeast and liver, although quite similar for *L. casei,* were different for *S. lactis* (194). It may well be that additional compounds will be isolated from other natural sources with varying activity ratios to the test organisms *S. lactis* and *L. casei.* Apparently the use of these two test organisms is a biological tool for establishing the differences in such compounds. It is possible that the use of other microorganisms and/or animals may also provide further differentiation. Evidently all these compounds and those already isolated have some common basic chemical structure since they are so closely related biologically. Since these compounds have been isolated from different sources, the name "folic acid," because of its etymological significance, should not be applied indiscriminately but should be reserved for the compound present in leafy materials.

As described in the section on sulfonamides, the granulocytopenia produced in rats by the feeding of diets containing sulfonamides could be overcome by the administration of norite eluate factor or folic acid concentrates. The administration of three crystalline preparations, two from liver and another from an unnamed source, was found to be effective in correcting this blood dyscrasia. Although no minimum effective dose was reported, the preparations were active at daily dose levels of from 5 μg. to 20 μg. in curative experiments (197). The report that xanthopterin was effective in producing leucocyte response (198) has not been confirmed by other workers (197, 199). The possible role of xanthopterin in mammalian nutrition was suggested by the interesting results of producing *L. casei* factor activity by allowing rat liver slices to act on xanthopterin. These experiments also sug-

gest the possible chemical relation of the *L. casei* factor to xanthopterin (200). It is interesting to note here the reported identity of xanthopterin with the pterin in urine, uropterin (201).

p-Aminobenzoic Acid

The increased interest in this factor has resulted in the development of additional procedures for its determination.

A microbiological assay for *p*-aminobenzoic acid using a mutant strain of *Neurospora crassa* was described (202). Enzymatic hydrolysis or autolysis of natural products appeared to liberate incompletely this active substance since heating with 6 *N* sulfuric acid for one hour at 115.5° C. gave increased amounts (203). Several colorimetric methods have been studied. In one, diazotized thiamin is coupled with *p*-aminobenzoic acid to yield a colored product. This method has been applied to urine (204). In another, the method for sulfanilamide was applied (205).

p-Aminobenzoic acid was found to produce a growth response in chicks on a purified diet. It was believed that this effect was produced indirectly by stimulating the increased production of unknown factors by the intestinal flora (206). A similar explanation was suggested by the ability of *p*-aminobenzoic acid to overcome the injurious effect of inositol in the lactation of rats (207).

As a result of the findings that natural source materials exhibited high antioxidant activity in the linoleic acid-butter yellow system, the various components of the B complex were investigated. *p*-Aminobenzoic acid was found to be the only one to possess any significant activity; however, it could not account for all this activity in natural extracts which indicated the existence of unknown or unrecognized antioxidants in such preparations as rice bran extract and liver extract fractions (208).

That *p*-aminobenzoic acid is an essential metabolite for microorganisms was indicated by its synthesis by growing organisms in a medium lacking in this factor (209). The ability of various strains of *Staphylococcus aureus* to resist the action of sulfonamides was shown to be dependent on the amount of *p*-aminobenzoic acid synthesized by the strain. Sulfonamide-resistant strains of *Staphylococcus aureus* produced greater amounts (210). *p*-Aminobenzoic acid was shown to be one of the many compounds which inhibit the thyroid gland in young rats (211). It was also found to be a highly effective detoxicant for high lethal doses of arsenicals such as carbarsone, tryparsa-

mide, etc. (212). The effect of *p*-aminobenzoic acid on gray hair in human subjects was studied (213, 214). However, no specific relationship could be determined.

The Effect of Sulfonamides

The use of the sulfonamides in experimental diets for rats has previously been described. Since the original work with sulfaguanidine, other derivatives such as succinyl-sulfathiazole, sulfathiazole, and sulfanilamide (215) have been found effective in producing biotin deficiency and blood dyscrasias in rats (199, 216). More recently the anemia in chicks, characteristic of vitamin B_c deficiency, was produced by the addition of sulfonamides to the diet (192).

The use of biotin and liver extract fractions as supplements overcame the disturbances caused by these sulfonamides (217, 218). The experimental facts may be summarized by stating that under the influence of diets containing substantial amounts of a sulfonamide there is a higher requirement for biotin and the liver extract fraction containing the norite eluate factor (*L. casei* factor).

The explanation generally offered is that the sulfonamides have so affected the intestinal flora that much less of these two factors is available to the host as a result of reduced intestinal synthesis (217). Under these conditions, the number of *E. coli* in the feces and cecum was shown to decrease temporarily with a return to approximately normal level at five weeks. The total counts on the other hand remained constant, the enterococci replacing the coliforms during the time the *E. coli* were repressed (219). However, since the animals did not grow during the time the intestinal population was returning to normal, it was believed that the microorganisms which resisted the sulfonamides had lost the ability to synthesize these growth factors (219).

In studying the counteraction of the bacteriostasis of *L. arabinosus* produced by sulfapyridine, it was observed that the arrest of growth could be overcome not only by *p*-aminobenzoic acid and nicotinic acid, but also by liver extract preparations which showed properties similar to those reported for the norite eluate factor (220). This is especially interesting in view of the fact that this organism, *L. arabinosus,* is known to synthesize this liver extract factor. Of course, demonstration with the pure crystalline norite eluate factor is necessary to confirm the fact that this reversal of inhibition is not due to the presence of some other active substance or substances. Thus, in the case of

L. arabinosus as in that of rats, sulfonamides increase the requirement for a growth factor, normally synthesized or otherwise available in adequate amounts.

From the results with a limited number of human subjects it appears that the intestinal synthesis of thiamin by the bacterial population might be significant (41). In these studies, the subjects were maintained on a highly purified and restricted ration. Some of these individuals failed to show the usual clinical signs of thiamin deficiency. Determination of free thiamin in the feces of these subjects indicated the presence of substantial amounts of this vitamin. On administration of a sulfonamide, the thiamin content was found to disappear promptly. The importance of intestinal synthesis of this vitamin was further demonstrated by showing that thiamin can be absorbed from the intestinal tract (41).

The signs of pantothenic acid deficiency, such as achromotrichia, porphyrin-caked whiskers, and reduced pantothenic acid content of the liver, were produced in rats on a diet containing succinyl-sulfathiazole (221). While pantothenic acid itself was ineffective in correcting this condition, the inclusion of both biotin and norite eluate factor (*L. casei* factor) concentrates was effective (221). The data suggest that these two factors are necessary for the proper utilization of panthothenic acid. Other workers have been able to produce pantothenic acid deficiency in the rat by the administration of sulfapyridine (222). In this case sulfapyridine reversibly inhibited the action of pantothenic acid. The question now may be raised as to whether sulfapyridine and succinyl-sulfathiazole act by the same mechanism. *p*-Aminobenzoic acid was found to counteract the action of sulfamethyldiazine on *Plasmodium lophurae* infections in Pekin ducklings. On the other hand, *p*-aminobenzoic acid did not affect the action of sulfanilamide and sulfamethyldiazine on lymphogranuloma venereum in mice (223).

The administration of sulfonamides certainly produces changes in the intestinal flora and blood constituents, but there is evidence that it causes pathological changes which may not respond to the expedients of dietary change (224). Feeding either sulfonamides or thioureas to rats, mice, and dogs produced a hyperemia and enlargement of the thyroid gland (224). The thyroid-metabolic effect produced in rats on a stock diet was not overcome by the administration of various vitamins or liver. It was prevented by thyroxin at 1 μg. per 10 gm. of body weight (224).

The death rate of baby chicks receiving sulfaguanidine was doubled (to approximately 50 per cent) when a mixture of *p*-aminobenzoic acid, thiamin, and riboflavin, or a suspension of *E. coli* was administered (225). From these observations it was felt that tolerance of the sulfonamide may be related to activities of the coliforms in the intestines (225).

Other Factors Associated with the B Vitamins

Without the inclusion in this review of a section summarizing the aspects of current researches which in years to come will receive increasing attention, this report would be incomplete. The recognition by biological or microbiological evidence of the possible existence of an additional unknown factor constitutes the first chapter in the story of a vitamin. It follows that the care in obtaining the experimental evidence for the existence of the new factor or vitamin will play an important role in its further development.

The existence of two additional factors required by the chick was reported (226). They have been described as a feather development factor, assigned the term B_{10}, and a growth factor, denominated B_{11}. Both were soluble in water, adsorbed on norite and superfitrol at pH 3, and eluted with alcohol-ammonia-water mixture, and were partially separated by fractional alcohol precipitation (226). The need for at least one factor beyond the *L. casei* factor for the chick was confirmed (227).

Another factor, organic in nature, was found to be required by turkey poults for the prevention of perosis (228). It was adsorbable on fuller's earth, from which it could be eluted with ammonium hydroxide (228).

Although mice were raised to the fourth generation of offspring on a purified ration containing pure vitamins and fatty acids, there were indications of the inadequacy of the diet as demonstrated by subnormal growth and higher mortality during weaning (229). Several explanations were suggested such as (*a*) the lack of a nursing factor, (*b*) inadequacy of one or more of the known dietary factors, and (*c*) an unbalance of factors (229).

Rats, though they were found to grow and reproduce essentially normally on a synthetic diet, showed a specific tail dermatitis (230). Some factor was believed to be present in natural foodstuffs which produced prompt remission of this condition (230). Deficiency signs such as dermatitis, loss of hair, and generally unhealthy skin were

observed in puppies on a purified ration. Yeast extract or liver was effective in preventing these conditions (231).

One of the unknown factors, GPF-3, required by the guinea pig, maintained on a basal diet containing 25 per cent linseed oil meal (232), was further studied (233). The activity substance was readily adsorbed by the usual adsorbants and was not extracted from either acid or alkaline solution by ether. It was stable to alkaline autoclaving whereas acid autoclaving destroyed most of its activity (233).

Xanthopterin was found to restore hemocytopoietic function in a monkey maintained on the vitamin M-deficient diet (234) supplemented with heat-treated liver (235). It was believed that other unknown factors, presumably in the heated liver, were necessary to prevent vitamin M deficiency (235). Vitamin M was found to be present in liver extract and yeast residue (236).

"Vitamin B_4" deficiency in the chick (237) was prevented by the addition of the amino acids, arginine, glycine, and cystine to the ration (238). From this it appears that the term "vitamin B_4" no longer has any significance.

The existence of several new microbial factors was reported (239, 240). Certain strains of *S. lactis* were found to require an unknown substance which is present in yeast extracts. It was not precipitated by the usual heavy metals or adsorbed on materials such as charcoal or fuller's earth (239). Natural extracts were found to contain a factor which stimulated the early growth of *L. casei* and which appeared to have practically no effect in the usual three-day period used in assays with this organism. Some of the properties of this substance were given (240). Yeast extract was found in experiments with the various propionibacteria to give growth stimulation superior to that obtained with an eight-vitamin supplement (241).

Ascorbic Acid

Methods of assay.—Further developments have appeared in the problem of ascorbic acid assay methods. In view of the importance that vitamins have attained in the adequate maintenance of not only civilian but also military nutrition and in the management of disease and casualties, methods for the determination of the vitamin content of foods and tissue should be as highly developed as possible.

Several modifications of the test with 2,6-dichlorophenolindophenol were studied (242, 243, 244). In one the excess unreduced dye was extracted into chloroform and then measured colorimetri-

cally. By this method the difficulties encountered in highly colored or turbid extracts could be overcome (242). In another, the method was based on the rate of decolorization of the excess dye (243). The nature of the possible products which would interfere with determinations using this dye was investigated (244).

Methods which did not use this dye were also presented (245 to 249). An assay procedure which was applied to blood and urine was based on the measurement of the color produced by reaction of 85 per cent sulfuric acid on the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid (245). The possible interfering substances such as glucose were found to be present in too low a concentration in the blood and urine to affect the results significantly (245). A photosensitized reaction with methylene blue has been utilized in a method for the determination of the vitamin in blood and its constituents (246). Another method involving electrolytic reduction of the total ascorbic acid has been applied to foodstuffs (247). Still another method was based on the reducing action of ascorbic acid on ferridipyridyl (248). The reaction of *o*-dinitrobenzene with ascorbic acid and the color reactions of dehydroascorbic acid were studied (249).

The effectiveness of various acids in stabilizing vitamin C during extraction from plant material was tested (250). From these studies it appeared that oxalic and metaphosphoric acids were equally suitable (250). In metaphosphoric acid extracts the vitamin was found stable at 0° C. in the dark over a period of two days (251).

Distribution in foodstuffs.—Unless dehydrated vegetables and fruits were packed in oxygen-free containers, substantial amounts of ascorbic acid were destroyed (252). Assays of fruits and vegetables, raw and cooked as would be consumed, were reported (253). The loss of the vitamin in different methods of cooking a vegetable, such as broccoli, was studied (254).

Exposure of green tomatoes to sunlight increased their vitamin C content, whereas bruising lowered it (255). Sunlight does not, however, affect the vitamin C content of processed foods (256.) The distribution of the vitamin in the various portions of the peach was studied (257). Although the white potato has been considered as an important source of this vitamin in diets, the sweet potato does not contribute any significant amounts (258).

Further assay values for varieties of rose hips and evergreens were reported (259). The use of the latter has played an important role recently in the prevention of scurvy in Russia (260). Other winter

sources of this vitamin in Great Britain were reported to be available in the various cresses (261). The buffalo-berry was found also to be an excellent source (262).

Chemistry of ascorbic and dehydroascorbic acids.—The oxidized form of ascorbic acid, dehydroascorbic acid, was proposed as the form which produces inhibition of urease activity since cysteine removes this inhibition by reducing dehydroascorbic acid to ascorbic acid (263). *In vivo* and *in vitro* experiments showed that dehydroascorbic acid was readily converted to diketogulonic acid (264). Since dehydroascorbic acid was readily converted in the liver to ascorbic acid, the following scheme of reactions was proposed:

$$\text{ascorbic acid} \rightleftarrows \text{dehydroascorbic acid} \rightarrow \text{diketogulonic acid}$$

The kinetics of the oxidation of ascorbic acid to form hydrogen peroxide were studied (265). In the presence of copper inhibitors ascorbic acid did not produce its characteristic inotropic and systolic effect on the frog heart due to the insufficient hydrogen peroxide formed under these conditions (266, 267). Inhibitors of copper in the auto-oxidation of the vitamin were found to be substances which contained at least one acid group along with some coordinating group. Compounds such as cystine, uric acid, aspartic acid, and glutamic acid were found to be most effective (268).

Ascorbic acid and metabolism.—An interesting inverse relationship was observed between the tissue content and excretion of ascorbic and citric acids. By increasing the citric acid content of certain tissues in the rat by feeding citrates, there was a decrease in the content of ascorbic acid in those tissues. Feeding of ascorbic acid resulted in a decrease in the excretion of citric acid (269).

The probable importance of this vitamin in carbohydrate metabolism was indicated by the finding that the insulin content of the pancreas was markedly diminished in scorbutic guinea pigs (270). Normal carbohydrate utilization, glycogen storage, and intermediary metabolism of proteins were believed to be dependent, at least in part, on the presence of this vitamin in adequate amounts in the body (271). Alcohol was metabolized more slowly in deficient animals than in normal animals (272). The deposition of trypan blue in the livers of scorbutic pigs injected with this dye was regarded as further evidence of hepatic damage and altered metabolism (273).

Biological synthesis of ascorbic acid.—Dairy cows have been maintained for three to four years on a low vitamin C diet and have con-

tinued to excrete vitamin C. There was no decrease from normal in the vitamin content of the blood plasma and milk during this period (274). The ability of the mature chicken to synthesize ascorbic acid was found not to be affected by vitamin A deficiency as indicated by comparison of the ascorbic acid contents of livers and duodena (275).

Glucoascorbic acid.—The feeding of mice and cotton rats on a synthetic ration of glucoascorbic acid, a compound structurally related to ascorbic acid, produced a condition resembling scurvy in susceptible species such as the guinea pig (276). Although ascorbic acid itself was not effective in either preventing or curing this condition, the removal of the compound or the substitution of foodstuffs which are natural sources for ascorbic acid was effective. Araboascorbic acid did not produce this scurvy-like condition (276).

Miscellaneous observations.—The effect of prolonged vitamin C deficiency on the healing of fractures and muscle injuries in guinea pigs was studied (277). The breeding performance of horses was found to be related to the blood ascorbic acid values. Good breeding mares had a significantly higher value than difficult breeders (278). Epithelial sheets in tissue culture were found to require ascorbic acid to remain healthy and active (279). The importance of vitamin C in the healing of wounds has been reviewed (280). The presence of sulfanilamide was found not to affect the ascorbic acid content of the wound, retard its healing, or reduce the tensile strength of the scar (281). The possible relationship between the deficiency of this vitamin and peptic ulcers was suggested by the improvement obtained by the use of ascorbic acid and the observation that more instances of this disorder are found in the spring (282).

The administration of ascorbic acid in 1 gm. daily doses was found to produce objective and subjective improvement in a small number of cases of essential hypertension (283, 284). The possible relation of vitamin C deficiency to gingivitis was indicated (285). The use of from 300 to 600 mg. daily doses of the vitamin has been suggested in the treatment of necrotic gingivitis (286).

Deficiency of this vitamin has been related to skin disorders (8, 9, 287). Vitamin C has been found to enhance the spirocheticidal and trypanosomicidal activity of the metals, such as arsenic and bismuth, probably as a result of its reductive properties (290). The vitamin was not found to minimize the effect of lead absorption (291). The role of ascorbic acid in the secretion of intraocular fluid was studied (292).

The status of vitamin C nutrition in various groups of individuals has been reported (293 to 297). A method for assessing the level of dietary intake by determining the number of days necessary to establish saturation with doses of 700 mg. per 140 lbs. has been described (298). Instead of the original method using the three-hour period, it was found that by using the period between the fourth and fifth hours after dosing with ¾ gm. of the vitamin much time was saved in carrying out the tests. This method was found to give consistent results so that it was possible to distinguish between slight variations in ascorbic acid intake (299).

Vitamin P

The time has not come when a definite statement can be made as to the existence or significance of vitamin P. However, evidence is gradually accumulating which will help in the evaluation of this vitamin. A method involving the capillary resistance of guinea pigs (300) was used to assay various types of fruits and vegetables for vitamin P activity (301). Fruits were found to be the best sources; green leaves and roots were much less active, and seeds were found to be very poor. It was also observed that vitamin P activity does not parallel that of ascorbic acid during germination (301). A method (302) for the determination of flavones or quercitin-like substances was applied to foodstuffs. Lemon peel gave the highest values, 1.66 mg. per gm.; on the other hand, a colorless vegetable such as cauliflower contained none (303).

Vitamins—General

Assays and sources.—Investigations on the increase of the vitamin content of dehydrated sprouted seeds (304) indicate the important role this source can play in supplying quantities of various B vitamins in the diet. However, some seeds, such as the cereals, wheat and barley (305), showed a slightly decreased thiamin value on malting. The increase in vitamin content during sprouting appears to be characteristic of the species (305).

The changes in the B content during development were also studied in beans and corn (306). The highest concentrations were found during relatively early stages followed by a steady decline as seeds mature (306).

Polished rice, well known for its lack of thiamin, also supplies inadequate quantities of pyridoxine, riboflavin, pantothenic acid, and

choline so that dry beriberi occurring on a rice diet is probably caused by complicated vitamin deficiencies involving these vitamins (307). The marked contrast in physique of inhabitants of Nigeria is probably attributable to their inclusion in a population group which subsists essentially on gari (derived from cassava) which is deficient in both thiamin and riboflavin, or on yams and sweet potatoes (308).

The importance of the addition of thiamin and riboflavin to white flour in human diets low in these two vitamins has received confirmation from animal experiments (309, 310). Enriched white bread with skim milk solids was found to be the equal of whole wheat in the promotion of growth and production of blood hemoglobin, and to be distinctly superior in the promotion of bone calcification. A combination of both skim milk solids and wheat berry residues gave the best growth stimulation (311). Peeled wheat flour and bread prepared from it have been assayed for their content of the various vitamins (312).

From experiments with adult human subjects over a period of nearly nine months in which the only variation in the diets consumed was in the bread, it was concluded that flour containing 2.0 mg. of thiamin and 15 mg. of nicotinic acid per pound with six parts per hundred of non-fat milk solids was a satisfactory food (313).

As a continuation of the study of the thiamin content (314) of the average American diet, the riboflavin, nicotinic acid, and pantothenic acid contents also were determined (315). On the basis of a 2,500 calorie daily intake, the values before enrichment were found to be 1.4 mg. riboflavin, 11 mg. nicotinic acid, and 4.9 mg. pantothenic acid. After enrichment, the riboflavin content was found to be 1.6 mg. and nicotinic acid, 17 mg. The value for pantothenic acid is substantially lower than the previously (316, 317) estimated requirement of 9 to 11 mgs. (315).

A revision (318) of the table of vitamin values of more than 150 foods has been published. The figures are based on data available prior to July, 1942.

Animal experiments.—The factors in rice polish extract, which were found necessary to maintain the normal epithelium in the forestomach of rats on essentially a flour diet, supplemented with riboflavin, nicotinic acid, and cystine, were found to be choline and pyridoxine (319). The influence of vitamin deficiencies on the incidence of congenital malformations in the offspring of rats maintained on B complex deficient (320) and riboflavin deficient diets was

studied (321). Dogs were observed to develop abnormal liver function on a B complex free diet (322). Although the increase of protein partially prevented the occurrence of disorders such as weight loss, moderate anemia, dermal and peptic ulcers, and fatty cirrhotic liver in dogs fed a low protein diet supplemented with thiamin, riboflavin, nicotinic acid, pyridoxine, and pantothenic acid, partial clinical improvement was effected by supplying large quantities of choline, powdered liver extract, or a "filtrate factor" prepared from rice bran extract (323). The combination of large amounts of choline and liver extract rapidly overcame these signs except the fibrosis of the liver (323).

An example of vitamin deficiency producing increased resistance rather than increased susceptibility to infection, in this instance spontaneous ulcerative cecitis, was observed in rats on a B complex deficient diet (324).

The influence of the various B vitamin deficiencies on the body composition of rats was investigated with results which showed differences between the sexes (115). Supplying thiamin produced in females an increase of the ratio of water, fat, and protein to residual substance whereas in males, thiamin increased only the ratio of fat to non-fat tissues (115). The effects of riboflavin, pyridoxine, and pantothenic acid were also reported (115). In studies of rats maintained on high and low levels of B complex, the concentration of riboflavin and nicotinic acid in the carcasses were the same, regardless of the level of vitamins fed or the level of protein in the diet (96). However, the liver contained higher concentrations of both riboflavin and nicotinic acid on the high protein diet, independent of the vitamin level. The thiamin content of the carcasses was dependent upon the intake of this vitamin irrespective of the protein level in the diet (96). In further studies on the effect of environment on the vitamin requirement of young rats, there was found to be no change in requirement for pyridoxine and riboflavin at higher temperatures (325), unlike the increased requirements for thiamin and choline (326) previously reported.

Growth stimulating properties for the rat have been ascribed to inositol and *p*-aminobenzoic acid when the thiamin intake was at a suboptimal level (327).

An instance in the interrelationship of the various vitamins was observed when dogs on a thiamin-free diet developed oral lesions which were healed by the administration of vitamin C (328). It

appears from this that vitamin B_1 was required by the dog to synthesize its normal vitamin C requirement (328). However, there exists the possibility of an increased vitamin C requirement in thiamin deficiency.

While the mammalian liver acts as a storage organ for various vitamins, the malpighian system of the American roach appears to have an analogous function (329). The distribution of the vitamins in the stages of maturity of the honeybee (67) were investigated. Experiments on the growth of five insect species showed that insects do not require any unknown or undetermined factors (330).

Clinical observations.—The use of vitamins in clinical neurology was summarized (331). The methods of therapy in vitamin deficiencies, symptoms and physical signs, and special laboratory tests for establishing and controlling these deficiency diseases have been discussed (332). The frequency with which the various signs and symptoms of vitamin deficiencies were observed in hospital patients was studied (333). Signs of deficiency were found present in all persons with hyperthyroidism, cirrhosis of the liver, and chronic alcoholism. They were also observed in two-thirds or more of those with infections, diabetes mellitus, carcinoma, and diseases of the gastrointestinal tract (333). The effect of vitamin therapy in infantile eczema (334) and Sydenham's chorea (335) appears to give in some cases favorable responses. The efficacy of iron therapy in hypochromic anemia (336) was reported to be unaffected by the addition of brewers' yeast.

There has been considerable interest in the possible losses of the various water-soluble vitamins in sweat (337 to 339). The conclusions generally reached are that while some thiamin, riboflavin, pantothenic acid, and nicotinic acid may be lost in sweat, these amounts are too small to be significant on an average dietary.

The various stages or states (340) of avitaminosis and the various methods of appraisal of these deficiency states have been described. By the methods which utilize gross and biomicroscopic examination of specific tissues for characteristic morphological changes, results were obtained indicating a high prevalence of malnutrition (340). Other studies (341) showed that the influence of early mild states of malnutrition prevented maximal work efficiency. The reduced work output of trained subjects on diets deficient in the vitamin B complex was returned to efficient levels by supplementation of the diet with B complex in the form of a yeast concentrate. Diets containing ap-

proximately one-third of the recommended levels produced subjective symptoms of easy fatigue, irritability, decreased interest and energy, anorexia, and increased leg pain during work (341). The extent to which the individual vitamin deficiencies are responsible would be of great value in judging the correctness of the recommended levels of these vitamins.

Vitamins and hormone activity.—Useful therapeutic effects of thyroid hormone are obtained by the simultaneous administration of extra amounts of the vitamins since the increased metabolism effected in experimental hyperthyroidism resulted in partial vitamin deficiencies (342).

The injection of 0.5 to 1.0 mg. of thyroxin in rats resulted in a substantial increase in the excretion of riboflavin while there was very little change in that of thiamin (343).

In contrast to the finding in female rats that in vitamin B complex deficiency the estrogen-inactivating function (344) of the liver was impaired, male rats with B complex deficiency showed no significant impairment of the ability of the liver to inactivate testosterone propionate (345). It appears, therefore, that in vitamin B complex deficiencies in man there may be an upsetting of the hormonal balance with an excess of estrogen resulting in disorders related to such conditions. This was demonstrated by the responses obtained in cases of menorrhagia, metrorrhagia, cystic mastitis, and premenstrual tension (346) when sources of vitamin B complex were administered.

LITERATURE CITED

1. Burk, D., and Winzler, R. J., *Science,* **97**, 57–60 (1943)
2. Williams, R. J., *Science,* **98**, 386 (1943)
3. Smith, M. I., *U.S. Public Health Repts.,* **45**, 116 (1930)
4. Various Authors, *U.S. Pharmacopoeia,* Eleventh decennial revision, 2nd Suppl., 129–32 (1939)
5. Chase, E. F., and Sherman, H. C., *J. Am. Chem. Soc.,* **53**, 3506–10 (1931)
6. Cowgill, G. R., *The Vitamin B Requirement of Man,* p. 33 (Yale University Press, New Haven, 1934)
7. Melnick, D., and Field, H., *J. Biol. Chem.,* **127**, 505–14, 515–30, 531–40 (1939)
8. Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.,* **61**, 179–83 (1939)
9. Brown, R. A., Hartzler, E., Peacock, G., and Emmett, A. D., *Ind. Eng. Chem., Anal. Ed.,* **15**, 494–95 (1943)
10. Vitamin B_1 Subcommittee of the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute, *Biochem. J.,* **37**, 433–39 (1943)
11. Platt, B. S., and Block, G. E., *Biochem. J.,* **37**, 439–43 (1943)
12. Allen, D. I., *J. Nutrition,* **25**, 521–37 (1943)
13. Reedman, E. J., and Young, G. A., *Can. J. Research,* **21C**, 145–50 (1943)
14. Friedemann, T. E., and Kmieciak, T. C., *J. Lab. Clin. Med.,* **28**, 1262–68 (1943)
15. Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.,* **61**, 179–83 (1939)
16. Brown, E. B., Hamm, J. C., and Harrison, H. E., *J. Biol. Chem.,* **151**, 153–61 (1943)
17. Owen, P. S., Weissman, N., and Ferrebee, J. W., *Proc. Soc. Exptl. Biol. Med.,* **52**, 59–60 (1943)
18. Pearce, J. A., *Can. J. Research,* **21C**, 57–65 (1943)
19. Kik, M. C., *Cereal Chem.,* **20**, 103–9 (1943)
20. Niven, C. F., Jr., and Smiley, K. L., *J. Biol. Chem.,* **150**, 1–9 (1943)
21. Meiklejohn, J., *Biochem. J.,* **37**, 349–54 (1943)
22. Hutchinson, G. E., *Arch. Biochem.,* **2**, 143–50 (1943)
23. Miller, R. C., Pence, J. W., Dutcher, R. A., Ziegler, P. T., and McCarty, M. S., *J. Nutrition,* **26**, 261–74 (1943)
24. Beadle, B. W., Greenwood, D. A., and Kraybill, H. R., *J. Biol. Chem.,* **149**, 339–47 (1943)
25. Greenwood, D. A., Beadle, B. W., and Kraybill, H. R., *J. Biol. Chem.,* **149**, 349–54 (1943)
26. Spitzer, E. H., Coombes, A. I., Elvehjem, C. A., and Wisnicky, W., *Proc. Soc. Exptl. Biol. Med.,* **48**, 376–79 (1941)
27. Sealock, R. R., Livermore, A. H., and Evans, C. A., *J. Am. Chem. Soc.,* **65**, 935–40 (1943)
28. Deutsch, H. F., and Hasler, A. D., *Proc. Soc. Exptl. Biol. Med.,* **53**, 63–65 (1943)

29. Field, J. B., Elvehjem, C. A., and Juday, C., *J. Biol. Chem.*, **148**, 261–69 (1943)
30. Woolley, D. W., and Krampitz, L. O. (In press)
31. Sure, B., and Ford, Z. W., *Proc. Soc. Exptl. Biol. Med.*, **54**, 83–85 (1943)
32. Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, **149**, 285–89 (1943)
33. Tracy, A. H., and Elderfield, R. C., *J. Org. Chem.*, **6**, 54–62 (1941)
34. Robbins, W. J., *Proc. Natl. Acad. Sci. U.S.*, **27**, 419–22 (1941)
35. Hauschildt, J. D., *Proc. Soc. Exptl. Biol. Med.*, **49**, 145–47 (1942)
36. Wyss, O., *J. Bact.*, **46**, 483–84 (1943)
37. Keys, A., Henschel, A. F., Mickelsen, O., and Brozek, J. M., *J. Nutrition*, **26**, 399–415 (1943)
38. Williams, R. D., Mason, H. L., Power, M. H., and Wilder, R. M., *Arch. Internal Med.*, **71**, 38–53 (1943)
39. Williams, R. D., Mason, H. L., and Wilder, R. M., *J. Nutrition*, **25**, 71–95 (1943)
40. Holt, L. E., Jr., *Penna. Med. J.*, **46**, 451–58 (1943)
41. Najjar, V. A., and Holt, L. E., Jr., *J. Am. Med. Assoc.*, **123**, 683–84 (1943)
42. Silverman, I., *Am. J. Surg.*, **40**, 671–72 (1938)
43. Carleen, M. H., Weissman, N., Owen, P. S., and Ferrebee, J. W., *Science*, **97**, 47–49 (1943)
44. Ring, G. C., *Am. J. Physiol.*, **138**, 488–90 (1943)
45. Williams, R. D., and Kendall, E. C., *Arch. Internal Med.*, **72**, 185–95 (1943)
46. Williams, R. H., Egana, E., Robinson, P., Asper, S. P., and Dutoit, C., *Arch. Internal Med.*, **72**, 353–71 (1943)
47. Whitmore, W. H., *Am. J. Roentgenol. Radium Therapy*, **49**, 83–98 (1943)
48. Le Galley, D. P., and Harrisson, J. W. E., *Am. J. Pharm.*, **115**, 95–99 (1943)
49. Forbes, R. M., *Cornell Vet.*, **33**, 27–47 (1943)
50. Myers, R. E., *J. Missouri Med. Assoc.*, **40**, 38–41 (1943)
51. Portis, S. A., and Zitman, I. H., *J. Am. Med. Assoc.*, **121**, 569–73 (1943)
52. Govier, W. M., *J. Pharmacol.*, **77**, 40–49 (1943)
53. Patton, J. W., *Vet. Med.*, **38**, 262–64 (1943)
54. Needles, W., *J. Am. Med. Assoc.*, **121**, 914–16 (1943)
55. Jones, O. H., *Am. J. Obstet. Gynecol.*, **45**, 869–74 (1943)
56. Browne, F. J., *Brit. Med. J.*, **I**, 445–46 (1943)
57. Ward, R., Sabin, A. B., Najjar, V. A., and Holt, L. E., Jr., *Proc. Soc. Exptl. Biol. Med.*, **52**, 5–7 (1943)
58. Toomey, J. A., Frohring, W. O., and Takacs, W. S., *Proc. Soc. Exptl. Biol. Med.*, **54**, 153–54 (1943)
59. Loewe, S., Loewe, I., and Knox, R., Jr., *Am. J. Digestive Diseases*, **10**, 65–66 (1943)
60. Knott, E. M., Kleiger, S. C., and Torres-Bracamonte, F., *J. Nutrition*, **25**, 49–58 (1943)
61. Rosenbaum, M., and Bradley, J., *Med. Clinics N. America*, **27**, 431–39 (1943)
62. Wilder, R. M., *Med. Clinics N. America*, **27**, 409–18 (1943)

63. Andrews, J. S., *Cereal Chem.*, **20**, 3–23 (1943)
64. Selye, H., *J. Nutrition*, **25**, 137–42 (1943)
65. Shukers, C. F., and Day, P. L., *J. Nutrition*, **25**, 511–20 (1943)
66. Klopp, C. T., Abels, J. C., and Rhoads, C. P., *Am. J. Med. Sci.*, **205**, 852–57 (1943)
67. Hunt, C. H., Burroughs, E. W., Bethke, R. M., Schalk, A. F., and Gerlaugh, P., *J. Nutrition*, **25**, 207–16 (1943)
68. Philpot, F. J., and Pirie, A., *Biochem. J.*, **37**, 250–54 (1943)
69. Tisdall, F. F., McCreary, J. F., and Pearce, H., *Can. Med. Assoc. J.*, **49**, 5–13 (1943)
70. Machella, T. E., and McDonald, P. R., *Am. J. Med. Sci.*, **205**, 214–23 (1943)
71. Conners, C. A., Eckardt, R. E., and Johnson, L. V., *Arch. Ophthalmol.* (Chicago), **29**, 956–67 (1943)
72. Nippert, P. E., and McGinty, A. P., *J. Med. Assoc. Georgia*, **32**, 295–97 (1943)
73. Browne, D. C., and McHardy, G., *Southern Med. J.*, **36**, 32–41 (1943)
74. Vorhaus, M. G., Gompertz, M. L., and Feder, A., *Am. J. Digestive Diseases*, **10**, 45–48 (1943)
75. Morris, H. P., and Robertson, W. v. B., *J. Natl. Cancer Inst.*, **3**, 479–89 (1943)
76. Sherman, H. C., Ellis, L. N., and Zmachinsky, A., *J. Nutrition*, **25**, 153–60 (1943)
77. Williams, R. D., Mason, H. L., Cusick, P. L., and Wilder, R. M., *J. Nutrition*, **25**, 361–77 (1943)
78. Hausman, E., Rosner, L., and Cannon, H. J., *Cereal Chem.*, **20**, 82–86 (1943)
79. Allinson, M. J. C., *J. Biol. Chem.*, **147**, 785–91 (1943)
80. Lamb, F. W., *Ind. Eng. Chem., Anal. Ed.*, **15**, 352–55 (1943)
81. Melnick, D., and Oser, B. L., *Ind. Eng. Chem., Anal. Ed.*, **15**, 355–56 (1943)
82. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *J. Am. Chem. Soc.*, **65**, 992 (1943)
83. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, **15**, 471–75 (1943)
84. Greene, R. D., Black, A., and Howland, F. O., *Ind. Eng. Chem., Anal. Ed.*, **15**, 77–78 (1943)
85. Bovernick, M. R., *J. Biol. Chem.*, **148**, 151–61 (1943)
86. Najjar, V. A., and Wood, R. W., *Proc. Soc. Exptl. Biol. Med.*, **44**, 386–92 (1940)
87. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395–400 (1943)
88. Kodicek, E., and Wang, Y. L., *Nature*, **148**, 23–24 (1941)
89. Sarett, H. P., *J. Biol. Chem.*, **150**, 159–64 (1943)
90. Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, **150**, 401–6 (1943)
91. Rogosa, M., *J. Bact.*, **46**, 435–40 (1943)
92. Kligler, I. J., Grossowicz, N., and Bergner, S., *J. Bact.*, **46**, 399–403 (1943)

93. Briggs, G. M., Luckey, T. D., Teply, L. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **148**, 517–22 (1943)
94. Hughes, E. H., *J. Animal Sci.*, **2**, 23–26 (1943)
95. Smith, S. G., Curry, R., and Hawfield, H., *J. Nutrition*, **25**, 341–48 (1943)
96. Sarett, H. P., and Perlzweig, W. A., *J. Nutrition*, **25**, 173–83 (1943)
97. Handler, P., *Proc. Soc. Exptl. Biol. Med.*, **52**, 263–64 (1943)
98. Frankau, I. M., *Brit. Med. J.*, 601–3 (Nov. 13, 1943)
99. Neuwahl, F. J., *Lancet*, **2**, 245, 348–51 (1943)
100. Perdue, J. R., *Southern Med. J.*, **36**, 198–201 (1943)
101. Melton, G., *Brit. Med. J.*, **1**, 600–1 (1943)
102. Atkinson, M., *Ann. Internal Med.*, **18**, 797–808 (1943)
103. Hardwick, S. W., *Lancet*, **2**, 43–45 (1943)
104. Lundquist, N. S., and Phillips, P. H., *J. Dairy Sci.*, **26**, 1023–30 (1943)
105. Clark, M. F., and Lechycka, M., *J. Nutrition*, **25**, 571–84 (1943)
106. Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.*, **15**, 141–44 (1943)
107. Stokes, J. L., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, **150**, 17–24 (1943)
108. Siegel, L., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, **149**, 361–67 (1943)
109. Williams, R. J., Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub.*, **4137**, 24 (1941)
110. Bina, A. F., Thomas, J. M., and Brown, E. B., *J. Biol. Chem.*, **148**, 111–16 (1943)
111. Snell, E. E., and Guirard, B. M., *Proc. Natl. Acad. Sci., U.S.*, **29**, 2, 66–73 (1943)
112. Stokes, J. L., Foster, J. W., and Woodward, C. R., Jr., *Arch. Biochem.*, **2**, 235–45 (1943)
113. Lepkovsky, S., and Nielsen, E., *J. Biol. Chem.*, **144**, 135–38 (1942)
114. Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, **149**, 195–201 (1943)
115. Voris, L., and Moore, H. P., *J. Nutrition*, **25**, 7–16 (1943)
116. Mowat, J. H., Pilgrim, F. J., and Carlson, G. H., *J. Am. Chem. Soc.*, **65**, 954–55 (1943)
117. György, P., *Biochem. J.*, **29**, 741–59 (1935)
118. Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.*, **148**, 253–54 (1943)
119. Bird, F. H., Kratzer, F. H., Asmundson, V. S., and Lepkovsky, S., *Proc. Soc. Exptl. Biol. Med.*, **52**, 44 (1943)
120. Cravens, W. W., Sebesta, E. E., Halpin, J. G., and Hart, E. B., *Poultry Sci.*, **22**, 94–95 (1943)
121. Chick, H., Macrae, T. F., Martin, A. J. P., and Martin, C. J., *Biochem. J.*, **32**, 2207–24 (1938)
122. Hughes, E. H., and Squibb, R. L., *J. Animal Sci.*, **1**, 320–25 (1942)
123. Wintrobe, M. M., Follis, R. H., Jr., Miller, M. H., Stein, H. J., Alcayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Bull. Johns Hopkins Hosp.*, **72**, 1–25 (1943)

124. Bischoff, F., Ingraham, L. P., and Rupp, J. J., *Arch. Path.*, **35**, 713–16 (1943)
125. Wright, C. S., Samitz, M. H., and Brown, H., *Arch. Dermatol. Syphilol.*, **47**, 651–53 (1943)
126. Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc. Exptl. Biol. Med.*, **54**, 123–25 (1943)
127. Robbins, W. J., and Ma, R., *Proc. Natl. Acad. Sci., U.S.*, **29**, 172–76 (1943)
128. Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519–30 (1942)
129. Lepkovsky, S., and Parsons, D., *J. Biol. Chem.*, **149**, 281–84 (1943)
130. Neal, A. L., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, **15**, 654–57 (1943)
131. Neal, A. L., and Strong, F. M., *J. Am. Chem. Soc.*, **65**, 1659–60 (1943)
132. Wright, L., *J. Biol. Chem.*, **147**, 261–62 (1943)
133. Light, A. E., and Clarke, M. F., *J. Biol. Chem.*, **147**, 739–47 (1943)
134. Stokes, J. L., and Martin, B. B., *J. Biol. Chem.*, **147**, 483–84 (1943)
135. Frost, D. V., *Ind. Eng. Chem., Anal. Ed.*, **15**, 306–10 (1943)
136. Schenck, J. R., *J. Biol. Chem.*, **149**, 111–15 (1943)
137. Schenck., J. R., *Proc. Soc. Exptl. Biol. Med.*, **54**, 6–7 (1943)
138. Kaplansky, S., *Z. physiol. Chem.*, **158**, 19–21 (1926)
139. Snell, E. E., Chan, L., Spiridanoff, S., Way, E. L., and Leake, C. D., *Science*, **97**, 168 (1943)
140. Woolley, D. W., and White, A. G. C., *Proc. Soc. Exptl. Biol. Med.*, **52**, 106–8 (1943)
141. Unna, K., *Proc. Soc. Exptl. Biol. Med.*, **54**, 55–57 (1943)
142. Pollack, M. A., *J. Am. Chem. Soc.*, **65**, 1335–39 (1943)
143. Gillis, M. B., Heuser, G. F., and Norris, L. C., *J. Nutrition*, **26**, 285–92 (1943)
144. Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, **144**, 393–400 (1942)
145. Hills, G. M., *Biochem. J.*, **37**, 418–25 (1943)
146. Leonards, J. R., and Free, A. H., *J. Nutrition*, 25, 403–10 (1943)
147. Taylor, A., Pennington, D., and Thacker, J., *J. Nutrition*, **25**, 389–93 (1943)
148. Ralli, E. P., and Graef, I., *Endocrinology*, **32**, 1–12 (1943)
149. Kerlan, I., and Herwick, R. P., *J. Am. Med. Assoc.*, **123**, 391–93 (1943)
150. Marenzi, A. D., and Cardini, C. E., *J. Biol. Chem.*, **147**, 363–70 (1943)
151. Rhian, M., Evans, R. J., and St. John, J. L., *J. Nutrition*, **25**, 1–5 (1943)
152. Engel, R. W., *J. Nutrition*, **25**, 441–46 (1943)
153. Evans, R. J., *Poultry Sci.*, **22**, 266–67 (1943)
154. Evans, R. J., Rhian, M., and Draper, C. I., *Poultry Sci.*, **22**, 88–93 (1943)
155. Engel, R. W., *Proc. Soc. Exptl. Biol. Med.*, **52**, 281–82 (1943)
156. Gavin, G., Patterson, J. M., and McHenry, E. W., *J. Biol. Chem.*, **148**, 275–79 (1943)
157. Handler, P. and Bernheim, F., *J. Biol. Chem.*, **148**, 649–54 (1943)
158. Handler, P., *J. Biol. Chem.*, **149**, 291–93 (1943)
159. Forbes, J. C., *Proc. Soc. Exptl. Biol. Med.*, **54**, 89–90 (1943)

160. Abels, J. C., Kupel, C. W., Park, G. T., and Rhoads, C. P., *Proc. Soc. Exptl. Biol. Med.*, **54**, 157–58 (1943)
161. Folch, J., and Woolley, D. W., *J. Biol. Chem.*, **142**, 963–64 (1942)
162. Folch, J., *J. Biol. Chem.*, **146**, 35–44 (1942)
163. Woolley, D. W., *J. Biol. Chem.*, **147**, 581–91 (1943)
164. Gross, P., and Kesten, B., *Arch. Dermatol. Syphilol.*, **47**, 159–74 (1943)
165. Laszlo, D., and Leuchtenberger, C., *Science*, **97**, 515 (1943)
166. Hofmann, K., *Advances in Enzymology*, **3**, 289–313 (1943)
167. du Vigneaud, V., Melville, D. B., Folkers, K., Wolf, D. E., Mozingo, R., Keresztesy, J. C., and Harris, S. A., *J. Biol. Chem.*, **146**, 475–85 (1942)
168. Harris, S. A., Wolf, D. E., Mozingo, R., and Folkers, K., *Science*, **97**, 447–48 (1943)
169. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175–78 (1940)
170. Melville, D. B., Dittmer, K., Brown, G. B., and du Vigneaud, V., *Science*, **98**, 497–99 (1943)
171. du Vigneaud, V., Dittmer, K., Hofmann, K., and Melville, D. B., *Proc. Soc. Exptl. Biol. Med.*, **50**, 374–75 (1942)
172. Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, **142**, 913–20 (1942)
173. Stokes, J. L., and Gunness, M., *Proc. Soc. Exptl. Biol. Med.*, **54**, 28–31 (1943)
174. Kögl, F., and Ten Ham, E. J., *Naturwissenschaften*, **31**, 208 (1943)
175. Hertz, R., *Proc. Soc. Exptl. Biol. Med.*, **52**, 15–17 (1943)
176. Elvehjem, C. A., Schweigert, B. S., Nielsen, E., and McIntire, J. M., *J. Nutrition*, **26**, 65–71 (1943)
177. Shull, G. M., and Peterson, W. H., *J. Biol. Chem.*, **151**, 201–2 (1943)
178. György, P., and Rose, C. S., *Proc. Soc. Exptl. Biol. Med.*, **53**, 55–57 (1943)
179. György, P., and Rose, C. S., *Science*, **94**, 261 (1941)
180. Fraps, R. M., Hertz, R., and Sebrell, W. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 140–42 (1943)
181. Hertz, R., Fraps, R. M., and Sebrell, W. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 142–44 (1943)
182. Rhoads, C. P., and Abels, J. C., *J. Am. Med. Assoc.*, **121**, 1261–63 (1943)
183. West, P. M., and Woglom, W. H., *Science.*, **93**, 525–27 (1941)
184. Sydenstricker, V. P., Singal, S. A., Briggs, A. P., and De Vaughn, N. M., *Science*, **95**, 176–77 (1942)
185. Caldwell, F. E., and György, P., *Proc. Soc. Exptl. Biol. Med.*, **53**, 116–19 (1943)
186. Trager, W., *Science*, **97**, 206–7 (1943)
187. Cooperman, J. M., Waisman, H. A., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 250–54 (1943)
188. Elvehjem, C. A., and Waisman, H. A., *J. Nutrition*, **26**, 361–75 (1943)
189. Lazere, B., Thomson, J. D., and Hines, H. M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 81–82 (1943)
190. Hogan, A. G., and Parrott, E. M., *J. Biol. Chem.*, **128**, p. xlvi (1939)

191. Hogan, A. G., and Parrott, E. M., *J. Biol. Chem.*, **132**, 507–17 (1940)
192. O'Dell, B. L., and Hogan, A. G., *J. Biol. Chem.*, **149**, 323–37 (1943)
193. Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404–5 (1943)
194. Stokstad, E. L. R., *J. Biol. Chem.*, **149**, 573–74 (1943)
195. Stokes, J. L., Keresztesy, J. C., and Foster, J. W. (In preparation)
196. Keresztesy, J. C., Rickes, E. L., and Stokes, J. L., *Science*, **97**, 465 (1943)
197. Daft, F. S., and Sebrell, W. H., *U.S. Public Health Reports*, **58**, 1542–45 (1943)
198. Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **147**, 257–58 (1943)
199. Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F., *J. Biol. Chem.*, **148**, 721–22 (1943)
200. Wright, L. D., and Welch, A. D., *Science*, **98**, 179–82 (1943)
201. Koschara, W., *Z. physiol. Chem.*, **277**, 159–62 (1943)
202. Thompson, R. C., Isbell, E. R., and Mitchell, H. K., *J. Biol. Chem.*, **148**, 281–87 (1943)
203. Mitchell, H. K., Isbell, E. R., and Thompson, R. C., *J. Biol. Chem.*, **147**, 485–86 (1943)
204. Kirch, E. R., and Bergeim, O., *J. Biol. Chem.*, **148**, 445–50 (1943)
205. Eckert, H. W., *J. Biol. Chem.*, **148**, 197–204 (1943)
206. Briggs, G. M., Jr., Luckey, T. D., Mills, R. C., and Hart, E. B., *Proc. Soc. Exptl. Biol. Med.*, **52**, 7–10 (1943)
207. Sure, B., *J. Nutrition*, **26**, 275–83 (1943)
208. György, P., and Tomarelli, R., *J. Biol. Chem.*, **147**, 515–24 (1943)
209. Landy, M., Larkum, N. W., and Oswald, E. J., *Proc. Soc. Exptl. Biol. Med.*, **52**, 338–41 (1943)
210. Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, **97**, 265–67 (1943)
211. Astwood, E. B., *J. Pharmacol.*, **78**, 79–89 (1943)
212. Sandground, J. H., *Science*, **97**, 73–74 (1943)
213. Brandaleone, H., Main, E., and Steele, J. M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 47–49 (1943)
214. Eller, J. J., and Diaz, L. A., *N.Y. State J. Med.*, **43**, 1331–32 (1943)
215. Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, **98**, 20–22 (1943)
216. Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F., *J. Biol. Chem.*, **148**, 721–22 (1943)
217. Welch, A. D., and Wright, L. D., *J. Nutrition*, **25**, 555–70 (1943)
218. Ransone, B., and Elvehjem, C. A., *J. Biol. Chem.*, **151**, 109–15 (1943)
219. Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 276–79 (1943)
220. Teply, L. J., Axelrod, A. E., and Elvehjem, C. A., *J. Pharmacol.*, **77**, 207–14 (1943)
221. Wright, L. D., and Welch, A. D., *Science*, **97**, 426–27 (1943)
222. West, H. D., Jefferson, N. C., and Rivera, R. E., *J. Nutrition*, **25**, 471–77 (1943)

223. Seeler, A. O., Graessle, O., and Dusenbery, E. D., *J. Bact.*, **45**, 205–9 (1943)
224. MacKenzie, C. G., and MacKenzie, J. B., *Endocrinology*, **32**, 185–209 (1943)
225. Lewis, K. H., Ham, W. C., and Jensen, W. I., *Proc. Soc. Exptl. Biol. Med.*, **52**, 33–35 (1943)
226. Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **148**, 163–72 (1943)
227. Almquist, H. J., *Proc. Soc. Exptl. Biol. Med.*, **54**, 57–59 (1943)
228. Patrick, H., Boucher, R. V., Dutcher, R. A., and Knandel, H. C., *J. Nutrition*, **26**, 197–204 (1943)
229. Foster, C., Jones, J. H., Dorfman, F., and Kobler, R. S., *J. Nutrition*, **25**, 161–71 (1943)
230. Erskoff, B. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 41–43 (1943)
231. Lambooy, J. P., and Nasset, E. S., *J. Nutrition*, **26**, 293–302 (1943)
232. Woolley, D. W., *J. Biol. Chem.*, **143**, 679–84 (1942)
233. Elvehjem, C. A., Mannering, G. J., Cannon, M. D., Barki, H. V., and Hart, E. B., *J. Biol. Chem.*, **151**, 101–7 (1943)
234. Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L., *J. Exptl. Med.*, **68**, 923–40 (1938)
235. Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *Federation Proc.*, **2**, 72 (1943)
236. Saslaw, S., Wilson, H. E., Doan, C. A., and Schwab, J. L., *Science*, **97**, 514–15 (1943)
237. Elvehjem, C. A., Keenan, J. A., Kline, O. L., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, **103**, 671–85 (1933)
238. Elvehjem, C. A., Briggs, G. M., Jr., Luckey, T. D., and Hart, E. B., *J. Biol. Chem.*, **150**, 11–15 (1943)
239. Smith, F. R., *J. Bact.*, **46**, 369–71 (1943)
240. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **147**, 183–87 (1943)
241. Thompson, R. C., *J. Bact.*, **46**, 99–104 (1943)
242. Kirkpatrick, H. F. W., *J. Soc. Chem. Ind.*, **62**, 39–41 (1943)
243. Hochberg, M., Melnick, D., and Oser, B. L., *Ind. Eng. Chem., Anal. Ed.*, **15**, 182–88 (1943)
244. Enders, C., *Biochem. Z.*, **314**, 389–98 (1943)
245. Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, **147**, 399–407 (1943)
246. Butler, A. M., Cushman, M., and MacLachlan, E. A., *J. Biol. Chem.*, **150**, 453–61 (1943)
247. Gunther, E., *Biochem. Z.*, **314**, 277–84 (1943)
248. Koenig, R. A., *Ind. Eng. Chem., Anal. Ed.*, **15**, 181–82 (1943)
249. Fearson, W. R., and Kawerau, E., *Biochem. J.*, **37**, 326–29 (1943)
250. Ponting, J. D., *Ind. Eng. Chem., Anal. Ed.*, **15**, 389–91 (1943)
251. Mapson, L. W., and Mawson, C. A., *Nature*, **151**, 222–23 (1943)
252. Aykroyd, W. R., *Nature*, **151**, 22–23 (1943)
253. Olliver, M., *Chemistry & Industry*, **62**, 146–48 (1943)
254. Barnes, B., Tressler, D. K., and Fenton, F., *Food Research*, **8**, 13–26 (1943)
255. Wokes, F., and Organ, J. G., *Biochem. J.*, **37**, 259–65 (1943)

256. WOKES, F., *Nature*, **152**, 328–29 (1943)
257. SCHRODER, G. M., SATTERFIELD, G. H., AND HOLMES, A. D., *J. Nutrition*, **25**, 503–9 (1943)
258. SCOULAR, F. I., AND EAKLE, D. H., *Food Research*, **8**, 156–62 (1943)
259. HUNTER, G., AND TUBA, J., *Can. Med. Assoc. J.*, **48**, 30–32 (1943)
260. SHISHKIN, B., *Science*, **97**, 354–55 (1943)
261. CROWE, H. W., AND BRADFORD, E. A. M., *Nature*, **151**, 505 (1943)
262. KNOWLES, D., AND WILK, I., *Science*, **97**, 43 (1943)
263. QUASTEL, J. H., *Nature*, **152**, 215 (1943)
264. PENNEY, J. R., AND ZILVA, S. S., *Biochem. J.*, **37**, 403–17 (1943)
265. SILVERBLATT, E., ROBINSON, A. L., AND KING, C. G. *J. Am. Chem. Soc.*, **65**, 137–41 (1943)
266. KRAYER, O., *Proc. Soc. Exptl. Biol. Med.*, **53**, 51–52 (1943)
267. KRAYER, O., LINSTEAD, R. P., AND TODD, D., *J. Pharmacol.*, **77**, 113–22 (1943)
268. PETERSON, R. W., AND WALTON, J. H., *J. Am. Chem. Soc.*, **65**, 1212–17 (1943)
269. PURINTON, H. J., AND SCHUCK, C., *J. Biol. Chem.*, **148**, 237–43 (1943)
270. BANERJEE, S., *Nature*, **152**, 329 (1943)
271. BEYER, K. H., *Arch. Internal Med.*, **71**, 315–24 (1943)
272. JERVIS, C. A., *Quart. J. Studies Alcohol*, **3**, 533–40 (1943)
273. RUSSELL, W. O., AND CALLAWAY, C. P., *Arch. Path.*, **35**, 546–52 (1943)
274. WALLIS, G. C., *J. Dairy Sci.*, **26**, 401–8 (1943)
275. RUBIN, M., AND BIRD, H. R., *Poultry Sci.*, **22**, 53–55 (1943)
276. WOOLLEY, D. W., AND KRAMPITZ, L. O., *J. Exptl. Med.*, **78**, 333–39 (1943)
277. MURRAY, P. D. F., AND KODICEK, E., *Nature*, **151**, 395–96 (1943)
278. DAVIS, G. K., AND COLE, C. L., *J. Animal Sci.*, **2**, 53–58 (1943)
279. CHAMBERS, R., AND CAMERON, G., *Am. J. Physiol.*, **139**, 21–25 (1943)
280. BOWERS, W. F., *J. Lab. Clin. Med.*, **28**, 451–62 (1943)
281. JONES, C. M., BARTLETT, M. K., RYAN, A. E., AND DRUMMEY, G. D., *New Engl. J. Med.*, **229**, 642–46 (1943)
282. KOHN, B., *Brit. Med. J.*, **1**, 489 (1943)
283. DAVIS, N. S., AND POSER, E. F., *J. Am. Med. Assoc.*, **122**, 59 (1943)
284. DAVIS, N. S., AND POSER, E. F., *Clin. Med. Surg.*, **50**, 152–54 (1943)
285. STUHL, F., *Lancet*, **1**, 640–42 (1943)
286. KENT, B. S., *Lancet*, **1**, 642–45 (1943)
287. DENNIS, C. C., *J. Missouri Med. Assoc.*, **40**, 77 (1943)
288. GUEQUIERRE, J. P., *Arch. Dermatol. Syphilol.*, **47**, 432 (1943)
289. KESTEN, B. M., *New Engl. J. Med.*, **228**, 124–27 (1943)
290. HUSKIN, S. L., *Am. J. Digestive Diseases*, **10**, 170–74 (1943)
291. EVANS, E. E., NORWOOD, W. D., KEHOE, R. A., AND MACHLE, W., *J. Am. Med. Assoc.*, **121**, 501–4 (1943)
292. FRIEDENWALD, J. S., BUSCHKE, W., AND MICHEL, H. O., *Arch. Ophthalmol.* (Chicago), **29**, 535–74 (1943)
293. HARRIS, L. J., AND OLLIVER, M., *Nature*, **151**, 22 (1943)
294. LEWIS, J. S., STORVICK, C. A., AND HAUCK, H. M., *J. Nutrition*, **25**, 185–96 (1943)

295. Pijoan, M., Elkin, C. A., and Eslinger, C. O., *J. Nutrition*, **25**, 491–96 (1943)
296. Brown, A. P., Fincke, M. L., Richardson, J. E., Todhunter, E. N., and Woods, E., *J. Nutrition*, **25**, 411–26 (1943)
297. Harper, A. A., MacKay, I. F. S., Raper, H. S., and Camm, G. L., *Brit. Med. J.*, **I**, 243–45 (1943)
298. Harris, L. J., *Nature*, **151**, 21–22 (1943)
299. Atkins, W. R. G., *Nature*, **151**, 21 (1943)
300. Bacharach, A. L., and Coates, M. E., *Analyst*, **67**, 313–17 (1942)
301. Bacharach, A. L., and Coates, M. E., *J. Soc. Chem. Ind.*, **62**, 85–87 (1943)
302. Wilson, C. W., Weatherby, L. S., and Bock, W. Z., *Ind. Eng. Chem., Anal. Ed.*, **14**, 425–26 (1942)
303. Weatherby, L. S., and Cheng, A. L. S., *J. Biol. Chem.*, **148**, 707–9 (1943)
304. Burkholder, P. R., *Science*, **97**, 562–64 (1943)
305. Davis, C. F., Laufer, S., and Saletan, L., *Cereal Chem.*, **20**, 109–13 (1943)
306. Cheldelin, V. H., and Lane, R. L., *Proc. Soc. Exptl. Biol. Med.*, **54**, 53–55 (1943)
307. Vedder, E. B., *Am. J. Trop. Med.*, **23**, 43–47 (1943)
308. Moore, D. F., *J. Trop. Med. Hyg.*, **45**, 129–31 (1942)
309. Williams, R. D., Higgins, G. M., Mason, H. L., and Gatz, A. J., *J. Nutrition*, **26**, 347–59 (1943)
310. Higgins, G. M., Williams, R. D., and Mason, H. L., *J. Nutrition*, **25**, 229–38 (1943)
311. Mitchell, H. H., Hamilton, T. S., and Shields, J. B., *J. Nutrition*, **25**, 585–603 (1943)
312. Sealock, R. R., and Livermore, A. H., *J. Nutrition*, **25**, 265–74 (1943)
313. Williams, R. D., Mason, H. L., and Wilder, R. M., *J. Am. Med. Assoc.*, **121**, 934–35 (1943)
314. Williams, R. R., Lane, R. L., and Johnson, E., *J. Nutrition*, **23**, 613–24 (1942)
315. Cheldelin, V. H., and Williams, R. R., *J. Nutrition*, **26**, 417–30 (1943)
316. Williams, R. J., *J. Am. Med. Assoc.*, **119**, 1–3 (1942)
317. Gordon, E. S., *Biological Action of Vitamins*, p. 136 (University of Chicago Press, Chicago, 1942)
318. Munsell, H. E., *Milbank Mem. Fund Quart.*, **21**, 102–8 (1943)
319. Sharpless, G. R., and Sabol, M., *J. Nutrition*, **25**, 113–17 (1943)
320. Warkany, J., Nelson, R. C., and Schraffenberger, E., *Am. J. Diseases Children*, **65**, 882–94 (1943)
321. Warkany, J., and Schraffenberger, E., *Proc. Soc. Exptl. Biol. Med.*, **54**, 92–94 (1943)
322. Drill, V. A., Shaffer, C. B., and Leathem, J. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 328–30 (1943)
323. Fouts, P. J., *J. Nutrition*, **25**, 217–28 (1943)
324. Bloomfield, A. L., and Lew, W., *J. Nutrition*, **25**, 427–31 (1943)
325. Mills, C. A., *Arch. Biochem.*, **2**, 159–62 (1943)
326. Mills, C. A., *Am. J. Physiol.*, **133**, 525–31 (1941)

327. McIntire, J. M., Henderson, L. M., Schweigert, B. S., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **54**, 98–100 (1943)
328. Govier, W. M., and Grieg, M. E., *Science*, **98**, 216–17 (1943)
329. Metcalf, R. L., *Arch. Biochem.*, **2**, 55–62 (1943)
330. Fraenkel, G., and Blewitt, M., *Nature*, **151**, 703–4 (1943)
331. Aring, C. D., *Bull. N.Y. Acad. Med.*, **19**, 17–33 (1943)
332. Spies, T. D., *J. Am. Med. Assoc.*, **122**, 497–502 (1943)
333. Goldsmith, G. A., *Southern Med. J.* **36**, 108–16 (1943)
334. Harris, A., and Gay, L. N., *J. Allergy*, **14**, 182–84 (1943)
335. Schwartzman, J., and Grossman, L., *Arch. Pediat.*, **60**, 194–200 (1943)
336. Moore, C. V., Minnich, V., Vilter, R. E., and Spies, T. D., *J. Am. Med. Assoc.*, **121**, 245–50 (1943)
337. Cornbleet, T., Kirch, E. R., and Bergeim, O., *J. Am. Med. Assoc.*, **122**, 426–29 (1943)
338. Mickelsen, O., and Keys, A., *J. Biol. Chem.*, **149**, 479–90 (1943)
339. Tennent, D. M., and Silber, R. H., *J. Biol. Chem.*, **148**, 359–64 (1943)
340. Kruse, H. D., *J. Am. Med. Assoc.*, **121**, 669–77 (1943)
341. Barborka, C. J., Foltz, E. E., and Ivy, A. C., *J. Am. Med. Assoc.*, **122**, 717–20 (1943)
342. Korenchevsky, V., Hall, K., and Clapham, B., *Brit. Med. J.*, **I**, 245–47 (1943)
343. Sure, B., and Ford, E. W., Jr., *Endocrinology*, **32**, 433–36 (1943)
344. Biskind, M. S., and Biskind, G. R., *Science*, **94**, 462 (1941)
345. Biskind, M. S., and Biskind, G. R., *Endocrinology*, **32**, 97–102 (1943)
346. Biskind, M. S., *J. Clin. Endocrinol.*, **3**, 227–34 (1943)

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THE FAT-SOLUBLE VITAMINS[1]

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Despite the reduced research output in the fat-soluble vitamin field from our American laboratories due to wartime conditions, and the paucity of reports from laboratories abroad, more papers came to the attention of the reviewer than could be mentioned in the space available. Insofar as possible an attempt has been made to let the work of the year speak for itself by concentrating attention on those areas of research interest in which the largest numbers of papers have appeared. A review, by Drill (1), of thyroid function and vitamin metabolism in which reference is made to several of the fat-soluble vitamins has just recently appeared.

Vitamin A

Absorption from the intestinal tract.—The importance of an understanding of the mechanism of absorption of vitamin A and carotene from the intestinal tract is underscored by numerous studies of this phase of the fate of this factor in the animal organism.

The role of phosphatides in the absorption of vitamin A and carotene has been given attention (2, 3). Using a vitamin A–tolerance test (4), according to which the serum vitamin A is determined in the fasting individual at intervals following the ingestion of a test dose of vitamin A, a rise of 41 per cent was noted four hours after a test dose of 180,000 I.U. (2). When in addition lecithin was fed, either as a commercial product from soy beans or as a defatted lecithin, the rise in blood vitamin A was of the order of 200 per cent. Under these conditions the carotene of the blood remained unchanged, although a test dose of carotene was not fed. The lecithin effect is apparently that of improved absorption, although there is the possi-

[1] Journal series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Agricultural Biochemistry. The author takes this opportunity to acknowledge the assistance of Mrs. Thalia Gates Mehrhof in the preparation of the review.

bility of a mobilization from vitamin A depots in the body. Since pure lecithin was not fed there is still the question of the effect of cephalin and inositol present in the lecithin preparation. In the white rat (3), two U.S.P. units of vitamin A in the form of carotene failed to promote growth even though the diet contained 4 per cent of cottonseed oil. When 1 per cent of soybean phosphatides was added to the diet satisfactory growth was obtained. The addition of 0.3 per cent of soybean oil, the quantity contained in the 1 per cent of phosphatides, caused a lesser growth response, a result which emphasizes that the effect was due to the phosphatides. Substitution of cod liver oil for carotene under the same experimental conditions gave a growth response similar to that obtained with carotene, although cod liver oil alone promoted a better growth than carotene alone. The enhancing effect of phosphatides on the utilization of vitamin A and carotene remains to be explained. The like effect of α-tocopherol suggests that phosphatides may be antioxidants.

To determine the effect of vitamin A concentration on absorption from the gastrointestinal tract, vitamin A solutions were fed by stomach tube, in amounts of 300 mg. per 100 sq. cm. of body surface area, to animals which had been fasted for forty-eight hours (5). The quantities of the vitamin administered in single doses ranged from 114 to 950,700 I.U., approximately in multiples of ten. Immediately after administration, 92 to 100 per cent of the vitamin and practically 100 per cent of the fat, which served as the vehicle for the vitamin, were recovered from the intestinal tract. The absorption during a three-hour period per 100 sq. cm. of body surface per hour increased from approximately 5 I.U., when 100 I.U. were fed, to 10,000 I.U., when 950,000 I.U. were fed. The rate of absorption of the vitamin was found to be proportional to the concentration administered and was 2,000 times faster at the higher level. While in terms of units the absorption of 10,000 I.U. per 100 sq. cm. of body surface per hour appears large, by calculation this is of the order of only 3.3 mg. of the pure vitamin. The absorption of the fat vehicle in milligrams per 100 sq. cm. of body surface per hour varied from 3.1 mg. when the highest dosage of the vitamin was fed and 10,000 I.U. were absorbed per 100 sq. cm. of body surface per hour, to 39.8 mg. when the feeding level was of the order of 150 I.U. and the absorption only 4.2 I.U. Thus no evidence of a proportionality was found between the quantities of the vitamin and fat absorbed. To answer the logical question as to whether the vitamin disappeared from the gut as a result of bac-

terial action, the intestinal tracts of some of the animals were incubated for three hours at 37° C. That the same quantity was recovered as from controls is evidence that bacterial destruction did not take place.

The effect of intestinal motor activity has been studied. In patients with sprue and in normal persons who have been given atropine, records of motor activity of the small intestine show that the tone is greatly reduced (6). Evidence has been presented (7) that the absorption defects of sprue-like states depend, in part at least, upon the lowered tone. Vitamin A absorption tests conducted with human subjects revealed that the appearance of the vitamin in the plasma was delayed after atropinization. Inhibition of pancreatic and biliary secretions appears to account for some of the delay. Also it is possible that the decrease in intestinal motility may be partly responsible, although no direct evidence to support this hypothesis is provided by the data.

Absorption of vitamin A in certain clinical conditions has been given attention. When the fat-tolerance and the vitamin A–tolerance tests (4, 8) were used in the study of intestinal absorption in active sprue, a failure to absorb fat and vitamin A was demonstrated, while, in cases of extensive granulomatous jejuno-ileitis, despite extensive diseased areas of the intestinal wall, a fairly satisfactory absorption of fat and vitamin A was observed. In chronic ulcerative colitis (9), the plasma vitamin A level did not rise as high, following oral administration of the vitamin, as in normal controls, a difference probably due to decreased absorption, although the number of bowel movements may be a factor. Also, impairment of absorption of vitamin A was noted in intractable infantile eczema (10).

Storage.—In young chicks (11) it was possible to maintain initial liver storage of vitamin A when the ration contained 250 to 500 μg. of carotene per 100 gm. of body weight and the storage was markedly increased at the higher ration levels. Thus when the intake was increased 3.5 times the increase in liver content was of the order of tenfold. At the same time the total yellow pigment increase was of the order of fivefold. Two reports have dealt with the storage of vitamin A by the rat as influenced by age and food content (12, 13).

Hepatic function and vitamin A.—Further evidence of the importance of the liver as a storage organ for vitamin A and of the influence of liver storage on the level in the blood has been obtained from an extensive study of seventy-five cases at operation for various diseases

of the upper abdomen. When the liver was damaged, a low plasma level of vitamin A was noted and, in the absence of gross nutritional deficiency, a plasma vitamin A level of zero indicated severe liver damage (14). In liver damage the hepatic concentration of vitamin A was often found to be reduced. Although the fluorescence-microscopic pattern of the distribution of vitamin A is never regular, only in cases of hepatitis, cirrhosis, and obstructive jaundice with secondary hepatitis were severe irregularities noted. In hepatitis the low level of the vitamin in the blood may be accounted for by impairment of the release of vitamin A from the liver and of absorption from the digestive tract.

High-altitude physiology.—Of interest in relation to high-altitude physiology is the observation that 80 per cent of a group of 107 rats which had been fed carrots for a period of ten days survived an exposure of two hours to a simulated altitude of 30,000 feet, whereas only 20 per cent of a like group fed a colony ration survived the exposure (15). Thus the feeding of fresh carrots for some unknown reason provided marked protection.

Vitamin A preparations and hypertension.—A report that vitamin A is effective in reducing the blood pressure in experimental hypertension (16) has stimulated attempts to confirm the finding and to ascertain whether another substance in fish liver oils is the effective agent. A substance, other than vitamin A, appears to be present in body and liver oils of fish which is effective in reducing the blood pressure of hypertensive rats (17). These oils offer greater promise than kidney as a source of the active principle effective in hypertension. Similar studies with dogs (18) revealed that the antihypertensive effect is not due to vitamin A and that the effective substance is present in some vitamin A concentrates but not in others. However, doses of 100,000 to 400,000 U.S.P. units of vitamin A concentrate given daily for from five to ninety days, were ineffective in the treatment of essential hypertension in the human (19). The isolation and identification of this substance presents an important problem.

Vitamin A–hormone relationships.—Since both estrogenic hormone and vitamin A may individually affect epithelial structures, the question was raised whether their action is independent or whether some relationship exists between them. In the monkey a study of the oral mucous membrane and vagina showed that large daily doses of vitamin A had an ameliorative effect on the keratinization and hyperplasia produced by the estrogen Progynon B (α-estradiol benzoate). When large doses of vitamin A, which caused degenerative change in the gingivae,

were followed by administration of vitamin A and the estrogen, the vitamin effect was overcome as indicated by hyperplasia of the gingivae with restoration of tissue tone (20). Vitamin A deficiency is not an etiological factor in the development of simple goiter in the rat. Hyperfunction of the thyroid is apparently accompanied by an increased requirement for the vitamin, due possibly to an increase in metabolic rate. In thyroidectomized animals there is a decreased need. The utilization of carotene, as compared with vitamin A, in the cure of xerophthalmia is not adversely affected by a deficiency of thyroid hormone (21).

Experimental pathology.—Mellanby (22) has shown that in young dogs on diets low in vitamin A local overgrowth of certain skull bones causes compression, twisting, and lengthening of most cranial nerves. Extensive degenerative changes were noted principally in the sensory nerves, the motor cranial nerves for the most part not being affected. Optic nerve degeneration may be produced in part from direct pressure of overgrown bone, from increased intracranial pressure, and from a primary degenerative change originating in the retina itself. The results of this study are similar to those reported by Wolbach & Bessey for the white rat (23).

Amblyopia, which may occur in the human following the use of tryparsamide, may be due, in part, to a lack of vitamin A. This is suggested by the observation that rats on a diet suboptimal in vitamin A when treated with the drug showed degenerative changes in the optic nerve, whereas no degeneration occurred when there was an adequate supply of the vitamin (24).

In the study of the pathology of the vitamin A–deficient rat little attention has been given to the skin. Gross cutaneous signs, different from those previously accepted as characteristic of the deficiency, were observed by Sullivan & Evans (25) in young rats on a diet deficient only in vitamin A, insofar as it is possible to devise such a ration. It is concluded that in previous studies vitamin A deficiency was complicated by deficiencies of the vitamin B complex, fat, and essential fatty acids. Attention is called to the desirability of the revision of the present vitamin A–deficient diet of the *U. S. Pharmacopoeia* if an uncomplicated deficiency is to be obtained. It should be pointed out, however, that the plateauing of growth and the appearance of deficiency symptoms occurred later in the more complete diets used by these investigators than when the *U. S. Pharmacopoeia* diet was used. Thus the animals would be heavier at the beginning of the assay period

and therefore possibly might not be as sensitive to differences in the vitamin A potency of dosage levels as animals of lower weight. The results, however, are of interest and perhaps the *U. S. Pharmacopoeia* diet should be reexamined in the light of these findings. In another study dealing with the skin, follicular hyperkeratosis leading to the formation of keratotic plugs in the hair follicles has been described as a characteristic lesion of the skin of rats deficient in vitamin A. Histologically and etiologically the keratotic plugs are completely homologous with those reported in man (26).

In acute avitaminosis A of short duration, retinas of rats show no structural changes except general edema. After a longer period of deprivation, degeneration begins in the visual cells and progresses through the outer nuclear layer, the pigmented epithelial layer, the outer molecular layer, and the inner nuclear layer. Slight degeneration of the outer segments of rods shows rapid repair under vitamin A therapy but if complete degeneration has taken place ten to eighteen weeks are required for repair. If the lesions involve the greater part of the outer nuclear layer they are irreparable (27).

The incremental growth rate of the incisal dentine of rats on a diet deficient in vitamins A and D is greatly reduced and a characteristic striation appears in the dentine (28).

Color vision and dark adaptation.—Since somewhat more than a million men of draft age are said to show some degree of color deficiency, the importance of salvaging at least some of this manpower is evident and further studies have been made of the effectiveness of vitamin A in the treatment of this deficiency. In one instance (29) a total of 1,400,000 U.S.P. units of vitamin A was administered to 41 subjects in an eight-week period but no significant improvement in color sensitivity was observed. In another study (30), in which 30,000 U.S.P. units of the vitamin were administered daily to four subjects for a ten-week period, an improvement of 20.7 per cent was noted as compared with 2.2 per cent in a control group. When 8 mg. per day of vitamin B_1 were used instead of vitamin A the improvement was 22.3 per cent. Under the same conditions the use of 16 mg. per day of vitamin B_2 resulted in an improvement of only 2.5 per cent. After a plateau had been reached, the vitamin A group was changed to the vitamin B_1 regime for a period of ten weeks. This resulted in an additional improvement of 11.8 per cent. When a change was made from the vitamin B_1 to the vitamin A regime the additional improvement averaged 10.8 per cent. It is apparent that the vitamin

therapy tried does not hold promise of success, although the improvement noted in one investigation may give rise to an extension of the study to include other essential dietary substances, such as the amino acids. It would be of interest to determine whether defective color vision is an hereditary metabolic defect or one that has been acquired during the life of the individual and whether, in either case, a reversible metabolic process is involved.

A further attempt has been made to determine what constitutes subclinical vitamin A deficiency in the human (31). For this purpose five young adults were examined with respect to the following: dark adaptation, determined by the biophotometer, the adaptometer, and regenometer (32); vitamin A and carotene of the blood; total white blood cell counts and differential blood counts; and changes in the structure of the skin and conjunctiva. For the study, three young women lived for 7.5 months and two young men for 4.5 months on a diet low in vitamin A, which supplied 67 to 100 U.S.P. units of the vitamin per day. None of the data obtained indicated any definite changes from observations made prior to the experimental period. The investigators conclude "that these subjects had sufficient stores to withstand the effects of the depletion diet or that these signs are not merely the result of uncomplicated vitamin A deficiency."

When a determination was made of the reliability of the Hecht adaptometer test and of its validity as a measure of the nutritional status of children as to vitamin A it was found that this procedure either does not measure first signs of a deficiency or that in the area studied (Chicago) a deficiency of this vitamin is not common (33). Since a welder sees a small bright area, particularly in arc welding, in a dark field, the question was raised as to whether, although provided with protection from actinic rays, a depletion of visual purple results which might retard adaptation to variations in brightness. On the day shift, welders who received 10,000 U.S.P. units of vitamin A before starting work and at the middle of the shift showed no greater gain in dark adaptation than those who did not receive a supplement. At night, however, a significant gain in dark adaptation was observed which was attributed to a greater demand for dark adaptation when not actually welding (34). Yudkin and associates (35), using a modified Crookes dark adaptation apparatus, have obtained complete dark adaptation curves for about 400 apparently normal subjects and have discussed the findings critically. Various psychological states have been found to affect dark adaptation readings (36).

Clinical observations. — When the rate of dark adaptation, the thresholds of the completely dark-adapted eye, and the blood vitamin A level were used as criteria, no difference was found between 20 patients with urolithiasis and normal controls. Furthermore in 78 autopsy cases with urolithiasis, the respiratory and urinary tracts did not show the epithelial metaplasia characteristic of vitamin A deficiency. Despite the observation that a vitamin A-free diet results in extensive epithelial changes in experimental animals, which may be accompanied by urinary calculi, there is as yet no positive proof that vitamin A deficiency of the subclinical type is an etiological factor in urolithiasis in man (37). In pneumonia, the vitamin A, carotene, and total lipid levels of the serum are reduced. Children over two years of age, during convalescence, showed a rise of serum lipid well above normal and, in general, the curve of serum vitamin A paralleled that of the lipids. In younger children, however, a vitamin A supplement was necessary to obtain a rise in blood lipids similar to that of older children, but there was little or no tendency toward such a rise in infants under eight months of age (38).

An investigation of the blood plasma level of vitamin A and carotene during pregnancy revealed a value of 105.4 I.U. (S.D. 32.2) of vitamin A per 100 cc. at six months or less and a significantly lower value of 91.1 I.U. (S.D. 26.2) in the third trimester. During the sixth, seventh, and eighth months the mean plasma carotene content was 145.9 μg. per 100 cc., a value significantly higher than that of 111.9 μg. for the first five months. Storage in the fetal liver and utilization by fetal tissues were suggested as causes of the lower vitamin A value in the third trimester. Also the possibility of an interference with release of vitamin A from the maternal liver in late pregnancy was suggested (39).

In another investigation the average vitamin A content of the cord blood of 50 infants was 91.3 I.U. per 100 cc. as compared with a maternal level of 106.3 I.U. obtained a few hours before delivery. In contrast, the average carotene content of the cord blood was 2.01 μg. per 100 cc. when the maternal level was 106.3 μg. When large doses of vitamin A and of carotene were administered just prior to delivery, the maternal vitamin A and carotene levels were increased but those of the cord blood remained within the untreated range. The constant presence of true vitamin A in fetal blood suggests that the factor is required and utilized in fetal life, but whether it is transferred directly from the mother or is formed from transferred carotene is

not known. The patients observed in this study, a total of 297 women, showed no correlation between plasma vitamin A and such clinical phenomena as puerperal infection, abortion, premature labor, toxemia, or hemorrhage at term (40).

Of 100 patients affected with acne, who were treated with a daily supplement of approximately 100,000 I.U. of vitamin A from halibut liver oil and later from a molecular distillate, seventy-nine became free or nearly free from eruption and only three did not improve (41).

Spector, McKhann & Meserve have presented a review of the effects of disease on the absorption, storage, and utilization of vitamin A (42).

Human requirements.—Lewis & Haig (43), using the dark adaptation method, found the minimum vitamin A requirement for infants to be 25 I.U. per kilogram of body weight. Later this group of investigators (44) reported that the blood level of vitamin A is a more sensitive indicator of a deficiency than dark adaptation. Using blood vitamin A as the criterion, they now report (45) that the daily minimum requirements for infants under seven months of age is between 100 and 200 U.S.P. units per kilogram of body weight, values which are from four to eight times that arrived at by the dark adaptation technique.

Vitamin A and domestic species.—In vitamin A–deficient hens, the ascorbic acid content of the liver and duodenum was as great as when ample vitamin A was provided. In contrast with the findings in cattle (46), therefore, vitamin A deficiency does not interfere with ascorbic acid synthesis (47). Attention has been given by Deuel and associates to the effect of massive doses of vitamin A on the hen. The feeding of a high vitamin A diet to the hen caused a lowering of the carotenoid pigments in the blood and liver which was greater than that noted in the blood or milk of cows under similar dietary conditions. That the chicken does not store carotene is not due to lack of absorption but is due to the transformation of the pigment to nonchromogenic compounds (48). When there is a progressive increase in the quantity of vitamin A up to 200,000 I.U. per lb. of basal diet high in carotenoids, there is a corresponding decrease in the pigment content of egg yolk, blood, and liver. Also there was a decrease of pigment in the body fat when 100,000 I.U. of vitamin A was fed for over six months. Vitamin A of the yolk, liver, and body fat increased with dietary intake, whereas the serum level was essentially unchanged except for a slight increase at the highest level of intake (49). Excellent evidence has been

presented that carotene is efficiently converted to vitamin A by the hen and that the feeding of equivalent practical dietary levels of either carotene or vitamin A leads to an equivalent egg yolk potency, due almost exclusively to true vitamin A (50). In the present study a differentiation was not made between the conversion of α- and β-carotene to vitamin A. From the scientific standpoint a study of the conversion of these forms and of cryptoxanthin to vitamin A would be of marked interest.

The histopathology of anasarcous lesions in vitamin A–deficient cattle has been described (51). The vitamin A content of the diet of the cow has been found to affect in direct relationship the quantity of this vitamin in the fetal liver, although the liver content is low (52). From a study of the relation between the carotene content of the dairy ration and the development of spontaneous rancidity in milk, it was concluded that the beneficial effect of green feed is not due to its high carotene content and that the hydrolytic rancidity which occurs in milk near the end of gestation on a dry-feed ration does not result from the low carotene intake (53). These observations are not in agreement with those of earlier reports (54).

In an earlier study, Howell, Hart & Ittner (55) reported that symptoms of vitamin A deficiency in horses were accompanied by lameness and joint lesions. Hart, Goss & Guilbert (56) now report that vitamin A deficiency is not the cause of the joint lesions. They present the hypothesis that the lesions are of nutritional origin and suggest that the preventive factor or factors may be present in green grass or leafy hay.

Factors influencing the carotene content of foods.—Little correlation was noted between the size of tomatoes and the carotene content; also, wide variations in the supply of macronutrient elements caused only slight variations in the carotene content of the fruit. Differences between varieties were not marked. Ripe greenhouse fruit, produced either in the summer or winter, contained less carotene than that produced outside in summer. Green fruit ripened in storage showed a much lower carotene content than vine-ripened fruit (57). Limitation of micronutrients had no significant effect on the quantity of carotene in tomatoes (58). Dehydration of carrots caused a carotene loss of approximately 6 per cent (59). During the storage of quick-frozen vegetables destruction of β-carotene was noted but the practice of blanching before quick-freezing retarded the loss (60). Fortification of foods with carotene necessitates attention to stabilization. Of several

antioxidants tried only hydroquinone was effective. In solvent-extracted soybean meal, carotene is more stable than in expeller-extracted meal and soybean lecithin favored the stability of carotene in soybean flour. A carotene concentrate from alfalfa was more stable than crystalline carotene in many products (61). Of about one hundred antioxidant substances studied, diphenylamine was found to have the greatest stabilizing effect on carotene. Not enough is known concerning the physiological effects of the substance to permit a conclusion as to its possible use as an antioxidant in foods (62). Carotene destruction in milk fat, either in the absence or presence of light, was found to be accompanied by oxidation of the double bonds of fats. In the destruction of vitamin A an additional photochemical reaction is involved (63). Irradiation of milk with ultraviolet light to produce 400 U.S.P. units of vitamin D per quart did not reduce the carotene or vitamin A content (64).

Estimation of vitamin A and carotene.—Aside from a study of the fluorescence of vitamin A under ultraviolet radiation with the possibility in mind of using this property as a basis for a quantitative method, no new approaches to the problem of the rapid and accurate determination of vitamin A and carotene have been noted, although several modifications of existing methods, especially those for carotene, have been reported.

Fluorescence of vitamin A_1 and A_2 esters in alcoholic solution, but not the free vitamin A alcohol, first increases then decreases under ultraviolet radiation. Although both processes are photochemical in nature, the second one is impeded by flushing with inert gases (65). When the vitamin A content of beef liver, determined with the quartz spectrograph, was compared with the potency in International Units estimated by bioassay, the spectro-vitamin A expressed in International Units, ranged from 3 I.U. per μg. in liver containing more than 43 μg. per gm. to 0.7 I.U. per μg. in liver containing less than 3 μg. per gm. Similar observations were made with pork liver. A formula has been prepared for the calculation of vitamin A in terms of International or U.S.P. units when spectro-vitamin A and carotene are determined (66). Methylene chloride has been found preferable to ether for the extraction of oil from soupfin shark livers (67).

For U.S.P. Reference Cod Liver Oil No. 2, the average conversion factor, on the basis of the claimed content of 1700 U.S.P. units of vitamin A per gram, is 2280 for the unsaponifiable fraction and 2000 for the whole oil. Conversion factors for a number of fish liver

oils show a decrease of 20 to 14 per cent from those obtained when Reference Oil No. 1 was used (68). Observations of this type emphasize the need for a new reference standard, a subject which has received excellent treatment by Hickman (69).

For the extraction of carotene from dehydrated plant materials a mixture of 30 per cent acetone and 70 per cent Skellysolve B, followed by separation from interfering pigments on a column, is proposed (70). The reaction between xanthophylls and 85 per cent orthophosphoric acid has been found satisfactory for the separation of these pigments from carotene (71).

The importance of special care in the handling of extracts of plant materials is emphasized by the interesting observation that photochemical destruction of carotene occurs in the presence of chlorophyll in acetone or petroleum ether. Both light and chlorophyll are necessary for the reaction which has been found to be a function of time. Chlorophyll takes part in the reaction and does not act as a catalyst. Sodium cyanide partially inhibits the photochemical reaction and prevents the enzymatic destruction of carotene (72).

A method has been described for the determination of carotene oxidase in seeds (73). To determine carotene in vegetable oils without saponification, a petroleum ether solution of the oil is passed through an aluminum oxide column to separate the pigment from other chromogens. Carotene is eluted with 2 per cent acetone in petroleum ether (74).

Vitamin A_2.—In a further study of vitamin A_2, liver oil concentrates from *Esox lucius* have been prepared and, on the basis of absorption spectra, it is concluded that only vitamin A_2 is present. The reaction products of vitamin A_2 and anhydro-vitamin A_2 with antimony trichloride show a value of about 3.0 for the ratio of E (695 mμ) to E (620 mμ). According to bioassay the concentrate had a vitamin A potency of 47,500 U.S.P. units per gram. Only vitamin A_2 was found in the livers of the animals used for the assay (75).

Vitamin D

Mineral metabolism.—Adult male white rats on a diet very low in calcium and essentially vitamin D–free showed a negative calcium balance and their molar teeth became friable, although chemical analysis did not reveal any decalcification. Alveolar bone was decalcified, resorption of the alveolar crest occurred, and the teeth became loose

in their sockets. The incisor teeth were not affected. It is of interest that the average ash content of the femurs was not significantly lower than that of the femurs of control animals and whereas the average density of the humeri of the experimental animals was less, the bone volume was not altered. Osteoporotic bones cannot be distinguished from normal bones on the basis of calcium and phosphorus percentages but the weight of ash or calcium or phosphorus per unit volume may serve for this differentiation. Thus in the osteoporosis produced, bone substance was lost from the interior of the bone and there was a proportionate decrease in the mineral and organic portions (76).

In a series of papers on the effect of dietary calcium, phosphorus, and vitamin D on the utilization of iron in the rat, Fuhr & Steenbock (77, 78, 79) have shown that with limited iron as ferric chloride and optimal calcium, slightly less hemoglobin was formed when optimal phosphorus was supplied as phytic acid than when it was supplied as phosphate. Formation of hemoglobin and storage of iron were decreased when calcium was fed in excess. When the calcium and phosphorus content were optimal and the iron content controlled, the addition of vitamin D caused an increase in hemoglobin and in total body iron. Neither the amount of body iron nor the rate of hemoglobin formation was reduced in low calcium or low phosphorus rickets.

Further interest has been manifested in the comparative effect of vitamins D_2 and D_3 and of dihydrotachysterol on calcium and phosphorus metabolism in the chick. McChesney (80), using the production of hypercalcemia as the criterion, has found the ratio of effectiveness of vitamin D_3 to vitamin D_2 to be 16 to 1, which differs significantly from the ratio of antirachitic effectiveness which is frequently stated to be 30 or 40 to 1 as measured by the percentage of bone ash. Dihydrotachysterol is less effective than vitamin D_3 but about as effective as vitamin D_2 in producing hypercalcemia. Correll & Wise (81), in a comparative study in which percentage of bone ash was used as the criterion, report that 3 I.U. of cod liver oil (11.1 mg.), 100 I.U. (2.5 μg.) of calciferol (vitamin D_2), or 0.5 I.U. (16.6 μg.) of dihydrotachysterol (called also, A.T.10) met the daily requirements for normal mineral metabolism in the chick. Thus, when calculated as the ratio of antirachitic effectiveness the results confirm the vitamin D_3 : vitamin D_2 ratio of approximately 30 to 1 and the A.T.10: vitamin D_3 ratio is of the order of 6 to 1. Toxic manifestations became evident when cod liver oil was fed at about 5000 times,

the calciferol at about 100 times, and A.T.10 at about 10 times the levels necessary for normal mineral metabolism. Since A.T.10 increases serum calcium and lowers bone ash, its effect on calcium metabolism resembles the action of both vitamin D and parathyroid hormone, the same effects being shown to a lesser extent by calciferol (vitamin D_2). The toxic dose of cod liver oil containing vitamin D did not elevate the blood calcium. The results of these two experiments may be contradictory with reference to the hypercalcemic effect of cod liver oil vitamin D, but it is not possible to arrive at a conclusion in this respect because the experimental conditions differed and McChesney expressed antirachitic potency by weight of substance rather than by units. The latter investigator may have fed a higher unitage of the vitamin in the form of vitamin D_3 than it was possible for Correll & Wise to feed in the form of cod liver oil, since substances other than vitamin D in cod liver oil may have caused the toxic effect. It has also been shown that doses of vitamins D_2 and D_3, of equivalent antirachitic effect in the chick, when administered orally maintained normal calcium and phosphorus metabolism, whereas with an equivalent dose of dihydrotachysterol serum calcium was lower and calcium and phosphorus retentions were less (82).

The hypothesis that a high carbohydrate diet results in poor calcium retention and an increased susceptibility to dental caries has been subjected to experiment. In rats on a high carbohydrate–low calcium diet, the use of vitamin D increased the growth and the survival period, improved appetite, increased retention of calcium and the calcium content of bones. These effects were not manifest when a high protein diet containing vitamin D was fed (83). On the same regime, the structure of bones and teeth was improved on both the high protein–low calcium and the high carbohydrate–low calcium diets when vitamin D was provided. Without the vitamin, the teeth were characterized by wide uncalcified predentin and dentin which showed globular calcification. On the vitamin D–deficient diets, the bony structures showed active resorption and reformation with the production of fibrous marrow spaces. The spaces simulated the "osteitis fibrosa" of hyperparathyroidism (84).

Hormone relationships.—When rats are fed a diet of yellow corn alone, decreased activity, low food intake, and a constant state of diestrus occur. All of these effects are corrected by giving the animals access to a solution of pure vitamin D_3 in vitamin and sterol-free cottonseed oil. Ultraviolet irradiation caused a less marked corrective

effect and vitamin A acetate and a distillate of cod liver oil free of vitamins A and D were essentially without effect. It is suggested that vitamin D_3 may act indirectly through stimulation of the anterior lobe of the hypophysis (85). Because factors other than vitamin D_3 are lacking, or occur in only small amounts, in yellow corn and because even after access to this vitamin full growth did not ensue, it would be of interest to see what results would be obtained, especially with regard to the estrous cycle, with rats on a synthetic type of diet containing all known dietary factors except vitamin D_3.

Calcium in the diet does not influence the size of the thyroid gland in the rat, but along with vitamin D calcium chloride as contrasted with the carbonate can act as a goitrogenic agent. The chloride may cause some loss of iodine which is followed by an increase in thyroid weight when an excess of calcium is absorbed (86).

Massive doses of vitamin D.—Interest continues to be manifested in the effect of large doses of vitamin D preparations. Neither vitamin D_2 nor D_3 in doses large enough to cause loss of weight brought about an increase in blood pressure in the rat (87). Likewise in the normotensive dog vitamin D_2 failed to raise the blood pressure (88). Following the administration of a single dose of 20,000 U.S.P. units of vitamin D per kilogram of body weight to the dog, the vitamin was found in the blood for about three months when tuna liver oil was fed and for about five months when either Delsterol or irradiated ergosterol was used. Tuna liver oil caused a mild transitory rise in blood calcium, Delsterol an immediate rise followed by a return to normal within a week, and irradiated ergosterol a prolonged rise which lasted about two months. The single doses provided protection against rickets for twelve to fourteen months. When a single massive dose, 200,000 U.S.P. units per kilogram of body weight, was administered as Delsterol, immediate prostration occurred and calcium excretion was increased. After the same number of units was administered as irradiated ergosterol the effect was less pronounced; calcium excretion was not increased but serum calcium was raised more markedly. Less than 10 per cent of the vitamin administered could be accounted for in the tissues, although some did appear in the feces. Thus the question as to the real effect and the fate of massive doses of vitamin D preparations remains unanswered (89). There is a need for a critical study in which pure vitamins D_2 and D_3 are used rather than preparations of high potency. In two patients with atrophic spondylitis and in two with degenerative arthritis of the spine, there was no primary disturb-

ance of calcium and phosphorus metabolism. Massive doses of activated ergosterol (Ertron) caused an increase in urinary calcium and phosphorus in all patients but the effect on the calcium and phosphorus balance and the fecal excretion was not uniform. Demonstrable changes in the activity of the parathyroids or thyroid were not produced (90). Excess of vitamin D fed the normal or the partially nephrectomized rat did not cause a significant increase in parathyroid volume (91).

Mode of action of vitamin D.—Further evidence has been obtained on the mode of action of vitamin D by the feeding of single large doses of vitamin D to dogs along with doses of radioactive phosphorus (92). The results obtained support the theory that vitamin D exerts its effect by intensification of phosphorus turnover in bone, accompanied by hyperphosphatemia and decreased visceral phosphorus turnover, rather than through increased intestinal absorption of phosphorus.

Experimental rickets.—In a continuation of a series of studies on experimental rickets in rats (93, 94, 95), Dodds & Cameron (96) report that rachitic rats show a subnormal growth and that the growth of the leg bones and vertebral column is greatly retarded on the Steenbock-Black rachitogenic diet 2965. Administration of vitamin D resulted in an increase in weight and an acceleration of bone growth. Reduction in growth rate, as well as other features of rickets, is believed to be due to phosphorus deficiency.

Estimation of vitamin D.—A physical-chemical method for the determination of vitamins D in fish liver oils described by Ewing *et al.* (97) is a long step toward the solution of the problem of finding a substitute for, or at least an adjunct to, the expensive and time-consuming bioassay procedure. After separation of the vitamins D and sterols from vitamin A and other interfering substances by chromatographic adsorption, the extinction coefficient, $E^{1\%}_{\text{cm.}}$ at 500 mμ, is determined for the reaction product of the combined vitamins D and sterols with antimony trichloride reagent and, after another chromatographic adsorption, for the sterols alone. The vitamins D value is obtained by difference. Using a conversion factor the extinction coefficient calculated for the vitamins D may be expressed in U.S.P. units. A comparison of the vitamin D potency of 51 liver oils from various salt-water fish, determined by the physical-chemical method, with that obtained by the U.S.P. bioassay procedure shows a fairly close agreement for oils whose potency is 5000 U.S.P. units per gram or higher.

Less satisfactory results were obtained for weaker oils. Fritz & Halloran (98) as the result of a critical study of the A.O.A.C. (Association of Official Agricultural Chemists) chick assay method for vitamin D conclude that the mineral content and possibly the source of the vitamin B_1-B_2 complex in the basal ration affect the degree of calcification. Also in their hands the ashing of green (freshly dissected) bones permitted the interpretation of assays as accurately as the ashing of dry, fat-free bones.

Human requirements.—Much needed information concerning the vitamin D requirements of the young adult has been reported by McKay and associates (99). When young women on a well-selected diet (including milk, eggs, and salmon) were given approximately 500 I.U. of vitamin D per day there was little influence upon calcium retention and it was concluded that the diet provided for the needs of the young adult for this vitamin.

Vitamin E

Experimental vitamin E deficiency and metabolism.—In an excellent review, Pappenheimer (100) has pointed out that vitamin E plays an essential role in skeletal muscle metabolism in all species of mammals so far studied, including the common laboratory animals, and in ducklings. No satisfactory evidence has been presented, however, that vitamin E is essential for the integrity of the nervous system. The author believes that the most pressing problem is to define in chemical terms the part that the tocopherols play in the complicated chemistry of skeletal muscle.

Muscle and brain cholesterol increased significantly in vitamin E-deficient rats. In brain tissue the increase of free cholesterol was especially marked, so that in the deficient animals the esters represented about 5 per cent and in the positive control group 35 per cent of the total. The changes are essentially parallel to those that occur in the rabbit but are not as striking, possibly because of the slow development of the dystrophy in grown rats (101). Using the iron-α,α'-bipyridine colorimetric method, the tocopherol content of liver tissue of rats and rabbits on diets high in tocopherol, on commercial dog chow, and on diets deficient in vitamin E was found to be for rats 42.3, 22.1, 22.6, and for rabbits 86.8, 9.2, and 9.4 mg. per kg. The average values for muscle tissues were for rats 11.9, 7.5, 4.8, and for rabbits 28.1, 8.0, and 5.7 mg. per kg. Tocopherol was not found in the urine

of rats on a high intake of this substance and tocopherylquinone was not present in liver, muscle, or urine, but considerable tocopherol was found in the feces (102). Increased capillary permeability in chicks results from the presence of highly unsaturated fatty acids in a diet deficient in vitamin E but the rancidity of the fat is not responsible for these symptoms (103). Even in the absence of microscopic changes in muscle fibers, the *in vitro* consumption of oxygen by skeletal muscle of young rats from vitamin E–deficient mothers was higher than that of litter mates protected by synthetic α-tocopherol. Similar observations were made with chicks. In contrast to the increase observed in rat skeletal muscle, intake of oxygen by visceral tissue, in this case rat liver, could not be detected (104).

The vitamin E content of the diet did not affect significantly the extent and velocity of neuromuscular regeneration in the rat following crushing of the tibial nerve. Likewise in the guinea pig the course of regeneration was not affected even by excess doses of the vitamin (105). In prolonged tocopherol deficiency in the rat, a marked elevation of phosphorus occurs in bone and soft tissues at nineteen weeks of age. This observation is of special interest in arriving at a better understanding of the chemistry of dystrophic muscle, because the increase in phosphorus parallels to a considerable extent the increase in oxygen consumption, and phosphorylation and oxidation are associated in muscle metabolism. Also the low creatine content observed in dystrophic muscle and the accompanying creatinuria (106, 107) would suggest a release of phosphorus. A high level of vitamin intake caused an increase in bone phosphorus at all ages. In the soft tissues phosphorus was elevated in some instances but in others no change occurred (108). The prophylactic requirements of the rat for α-tocopherol for growth, reproduction, and maintenance of normal striated muscle are reported for a sixteen-month experimental period (109).

Patrick & Morgan (110) have reported the interesting observation that an unidentified, fat-soluble nutrient, present in yeast and in soybean phosphatides, is necessary for the utilization of α-tocopherol by the chick. This observation should be given further attention because the results may add considerably to an understanding of the role of vitamin E in the animal organism.

Clinical observations.—In amyotrophic lateral sclerosis and in miscellaneous myopathies, the serum tocopherol level averaged 0.67 and 0.61 mg. per 100 cc., respectively. When 75 to 740 mg. of tocopherol

was administered daily, the response in the blood serum ran parallel to the dosage, and levels of more than 2.0 mg. per 100 cc. of serum were noted. Temporary favorable effects on the clinical status were observed only when the dose exceeded 200 mg. of tocopherol per day (111). Continuing his study of the role of vitamin E in pregnancy, Shute (112) reports that in a group of pregnancies a large percentage of the threatened abortions and miscarriages, in which there were either severe pains or considerable bleeding, was averted by the administration of vitamin E. A deficiency of this vitamin appears to be very common in pregnant women.

Chemistry and properties of vitamin E.—As a further contribution in the series of outstanding papers on the chemistry of vitamin E, Smith & Sprung (113) report the synthesis of "phytol ketone" (6, 10,14–trimethyl-2-pentadecanone) in excellent yield. This ketone makes practicable the tocopherol synthesis of Smith & Miller (114) and obviates the necessity of a supply of phytol, which has been a limiting factor in the production of α-tocopherol. Natural α- and γ-tocopherols have been crystallized and certain properties determined, but attempts to crystallize natural β-tocopherol have not been successful (115). The isolation of α-tocopherol from Mangona shark liver oil is of interest because it shows that the tocopherols may function as natural antioxidants in fish liver oils as well as in oils of vegetable origin (116).

Estimation of vitamin E.—By a critical study of the bioassay procedure it has been shown that improved results are obtained by discarding rats when less than seven and more than fourteen implantations occur, that dosing prior to mating is less effective than after mating, that infanticide is eliminated if assay rats are autopsied prior to parturition, and that vitamin E activity is adversely affected by lard and cod liver oil in the basal diet during the dosing period (117). Further modifications have made possible the use of the iron-α-α′-bipyridine color reaction for the determination of the tocopherols in liver tissue, urine, and feces (102).

Vitamin K

Factors affecting prothrombin activity.—Considerable attention has been given to factors affecting prothrombin activity and certain of the results are of interest in relation to vitamin K investigations. Thus, in a further study of the use of dilute (12.5 per cent) plasma, increased

plasma prothrombin activity was observed in certain clinical conditions —phlebitis, post-operative surgical trauma, and during childbirth— whereas with undiluted plasma the established normal values were obtained. The existence of prothrombin "activators" is suggested in attempting to account for this difference in clotting activity (118). For peripheral arterial plasma the prothrombin time is generally slightly in excess of that of venous plasma, but the difference is so slight that arterial and venous blood may be used interchangeably (119). It is recommended that the prothrombin clotting time be determined as soon as possible after the blood is obtained because the clotting time has been found to increase in proportion to the time of storage and at a given temperature the increase is a straight-line function of the logarithm of the storage time (120). The influence of the age of the organism on the vitamin K requirement and prothrombin formation has been given attention and it is concluded that there is very little evidence that the chick, for example, requires an increase of vitamin K intake with age. On the other hand, the fact that young chicks require 1.0 to 2.0 μg. of 2-methyl-1,4-naphthoquinone per day to maintain a normal level of prothrombin may mean that this relatively large quantity of the factor may be necessary for the formation of prothrombin to meet the increase in body size and blood volume. Although the quantity of the factor necessary to maintain a normal prothrombin level is fairly high, only about one twentieth of this amount is needed to protect against the hemorrhagic condition. During the first few days of life, the prothrombin level was found to rise rapidly, after which there was only a very gradual upward trend (121). Neither cephalin nor saline extracts of muscle tissues affected the coagulative behavior of plasma from chicks deficient in vitamin K (122).

Intestinal synthesis of vitamin K.—The bacteriostatic effect of Sulfasuxidine in young cecectomized rats, on a vitamin K–free diet, is sufficient to cause a high incidence of severe hypoprothrombinemia which can be alleviated by the administration of 2-methyl-1,4-naphthoquinone. *p*-Aminobenzoic acid partially counteracts the inhibitory effect of Sulfasuxidine on vitamin synthesis in the intestinal tract. Although the cecum is the important site of vitamin K synthesis, the vitamin can be formed in other parts of the tract (123).

Clinical investigations.—Evidence is being accumulated that vitamins C and K and the mineral supply of the maternal organism may be vital factors in the early growth of the embryo. Furthermore, vita-

mins C and K may be important factors in certain cases of threatened and spontaneous abortion, and a deficiency of these vitamins, individually or together, may be a cause of certain cases of antepartum bleeding and abortion (124). It is well known that low prothrombin in infants at birth can be prevented by giving vitamin K to mothers daily by mouth for a period prior to delivery. Since it is difficult to know when to begin the protective dosage it would be of practical value to know how near delivery the vitamin could be given and still be effective. Thyloquinone (2-methyl-1,4-naphthoquinone) in oil administered to the mother after the beginning of labor, and at least four hours before delivery, was found to be effective in the newborn infant and for the first week or ten days of life. Hypoprothrombinemia in untreated infants shows a seasonal incidence, being more frequent in February and March (125).

Vitamin K-Dicumarol relationship.—In a series of well-planned and well-executed researches, Link and associates have isolated the causative agent of sweet clover disease in cattle (126) from spoiled sweet clover hay (*Melilotus alba*), have demonstrated that it is the dicoumarin—3,3′-methylenebis(4-hydroxycoumarin)—and have accomplished its synthesis (127). For convenience in this discussion the substance will be referred to by the trade-mark designation, Dicumarol. It is of interest in relation to vitamin K because the two compounds are antagonistic in their effect in the animal organism. Dicumarol does not affect the clotting power of either blood or plasma *in vitro* (126, 128). When ingested by the rat, Dicumarol induces hypoprothrombinemia which is counteracted by vitamin K, given either before the anticoagulant is fed, along with it, or twelve hours afterward (129). Likewise, single doses of salicylic acid will induce in rats a temporary hypoprothombinemia when the diet is low in vitamin K, in all respects like that caused by Dicumarol. When the vitamin is present in the diet hypoprothrombinemia does not develop as in the case of rats fed both Dicumarol and vitamin K (130). The degradation of 3,3′-methylenebis(4-hydroxycoumarin) to salicylic acid *in vitro* has been established (127, 131), but the group of investigators concerned with this study point out that

> the thesis that 3,3′-methylenebis(4-hydroxycoumarin) is metabolized in the body to salicylic acid and thereby exerts its hypoprothrombinemia-inducing effect obviously cannot be accepted until salicylic acid, a derivative of salicylic acid, or some definitive degradation product arising from either has been isolated from the tissue.

Further results of the study of these interrelationships and their bearing on an understanding of the mechanism of prothrombin formation will be awaited with interest.

The antagonistic effects just described have been demonstrated in man. Shapiro and associates (132) showed that synthetic vitamin K in relatively large amounts counteracts or neutralizes the hypoprothrombinemia caused by the smallest single effectual dose of Dicumarol. In tests on a small number of patients, however, Davidson & MacDonald (133) found that synthetic vitamin K did not serve as an antidote. But more recently, these investigators (134) reported that as a result of an attempt to administer sufficient vitamin K or vitamin K–like substance in order to control large doses of Dicumarol, vitamin K_1 oxide was found to be effective in reversing the hypoprothrombinemia without serious toxic manifestations. Studies in man confirm the observations mentioned above with regard to the rat, that salicylates induce a hypoprothrombinemia which can be prevented by vitamin K (135, 136).

Phthalic acid and vitamin K activity.—Shemiakin, Schukina & Shvezov (137) offer the hypothesis that the natural vitamins K and their synthetic analogues are provitamins and that their antihemorrhagic activity results from an oxidative decomposition to phthalic acid in the animal organism. The latter compound is proposed as the effective antihemorrhagic agent. The conversion of highly active analogues of vitamin K to phthalic acid by heating with water and the observation of these investigators that the diethyl ether of phthalic acid has high antihemorrhagic activity have led to the proposed hypothesis. Dam (138), however, reports that neither potassium acid phthalate nor diethyl phthalate shows any antihemorrhagic activity.

Miscellaneous.—A more complete study of the vitamin K_1 absorption curve has revealed a new maximum at 239 mμ. For pure vitamin K_1, $E^{1\%}_{1\,\text{cm.}}$ at 249 mμ. is 435 ± 5. In hexane solution, at room temperature in the dark, the vitamin was found to be stable for as long as five months but it is decomposed rapidly by ultraviolet light, the point of attack probably being through the quinone group. Visible and infrared radiations had no effect (139).

Excess vitamin K, administered to dogs in which the intima of veins had been traumatized mechanically, did not increase the incidence of thrombosis (140). Neither the inorganic and lipid phosphorus nor the calcium of the plasma of vitamin K–deficient chicks differed significantly from the normal (141).

The antihemorrhagic activity of honey, equal to approximately 0.25 μg. of 2-methyl-1,4-naphthoquinone per gram, was greatly reduced when fed to chicks in an aqueous solution. The activity of the quinone was also reduced when fed in aqueous solution but was restored when ethyl laurate was the solvent. Likewise ethyl laurate restored the activity of alfalfa administered in aqueous suspension (142).

A New Fat-Soluble Dietary Factor

In a group of papers published in 1935 and 1936, Wulzen & Bahrs (143, 144, 145) reported their studies of the effect of certain foods in the diet of the guinea pig on the nutritive quality of certain tissues of this species for planarian worms. When fresh grass or kale was added to a basal diet, designed to be adequate except for vitamin C, guinea pig tissues from animals on this diet had satisfactory nutritional properties for the worms. On the other hand if orange juice or tomato juice was fed in place of the green food, the feeding of liver, heart, and kidney tissue produced severe dietary disease in planaria. The tissues became deficient for planaria after guinea pigs had been on the diet without green food for one month although the animals themselves had a normal appearance. If, however, the guinea pigs were allowed to remain on the diet for several months a deficiency developed which always proved fatal. These studies led to the conclusion that a substance is present in fresh kale and fresh alfalfa which is essential for planarian and mammalian nutrition. Apparently no attempt has been made to isolate the factor in green plant tissue.

Several years later, in 1941, these investigators (146) reported that guinea pigs fed raw milk with an addition of skim milk powder, copper and iron salts, carotene, and orange juice grew well and showed no abnormalities at autopsy. When pasteurized whole milk was used, deficiency symptoms began to appear, wrist stiffness being the first sign. The substitution of skim milk for whole milk intensified the deficiency which was characterized by great emaciation and weakness before death. The animals remained in normal posture and showed little tendency toward paralysis of the limbs. At autopsy the muscles were found to be extremely atrophied, and closely packed, fine lines of calcification ran parallel to the fibers. Also calcification occurred in various other parts of the body. When cod liver oil replaced carotene in the skim milk diet, paralysis developed quickly. The feed-

ing of raw cream cured the wrist stiffness. The deficiency symptoms on the milk diets are not identical with those induced by a lack of green food in the diet, although the two deficiencies may have some features in common. In a continuation of the study of the factor in raw cream, van Wagtendonk & Wulzen (147) reported in 1943 that vitamin E would not cure or prevent the deficiency, although the symptoms resembled those due to a lack of this vitamin, and the "grass juice factor" (148) was also ineffective. Gouley (149) reported that raw cream contained methyl vinyl ketone and that this substance was the curative factor, but the presence of the ketone was not confirmed by van Wagtendonk & Wulzen. A dosage of 5 mg. of the synthetic ketone per day for six days cured the wrist stiffness but was very toxic.

Using the disappearance of wrist stiffness as the criterion, van Wagtendonk & Wulzen have defined one guinea pig unit (U.) as follows: "a solution of an active fraction in Wesson oil contains one unit per cc. if, when 1 cc. is administered daily for five consecutive days to a sick animal, it cures the affected animal in this time . . . ." The cure refers to the disappearance of wrist stiffness.

Starting with 15 gallons of raw cream whose potency was 1 U. per gm., and containing a total of 51,000 U., these workers obtained 3 mg. of concentrate whose potency was 10,000,000 U. per gm., a recovery of 30,000 of the original 51,000 U. The steps of the isolation procedure included saponification and extraction with ether and were of such a nature as might be expected to yield a substance with fat-soluble properties. The operations were carried out in an atmosphere of nitrogen because heating cream in the presence of oxygen destroys the factor.

The substance is reported to have a molecular weight of about 200 and to contain a carbonyl group, although on the basis of the papers available to the reviewer the evidence for these conclusions is not clear.

Because of the rapid advances which have been made in our knowledge of the role of specific substances in mammalian nutrition, it would seem advisable to determine whether the deficiency symptoms appear when guinea pigs are fed a diet of the synthetic type in which all of the known essential nutrients have been supplied in liberal quantities and whenever possible as chemical entities. Since the symptoms in the guinea pig resemble those due to a lack of tocopherol, although this factor did not cure or prevent the deficiency disease, attention should be given to the recent report by Patrick & Morgan (110) that an

unrecognized fat-soluble substance is present in yeast and in soybean phosphatides which is necessary for the proper utilization of vitamin E by the chick.

Whether the dietary essential reported by Wulzen & Bahrs to be present in green food and that in cream are identical is yet to be demonstrated.

LITERATURE CITED

Vitamin A

1. Drill, V. A., *Physiol Revs.*, **23**, 355–79 (1943)
2. Adlersberg, D., and Sobotka, H., *J. Nutrition*, **25**, 255–64 (1943)
3. Slanetz, C. A., and Scharf, A., *Proc. Soc. Exptl. Biol. Med.*, **53**, 17–19 (1943)
4. Kann, S., and Sobotka, H., *Abstract 104th Meeting Am. Chem. Soc.*, p. 11 B (Buffalo, N.Y., Sept., 1942)
5. Reifman, A. G., Hallman, L. F., and Deuel, H. J., Jr., *J. Nutrition*, **26**, 33–42 (1943)
6. Ingelfinger, F. J., Moss, R. E., and Helm, J. D., Jr., *J. Clin. Investigation*, **22**, 699–705 (1943)
7. May, C. D., and McCreary, J. F., *J. Pediat.*, **17**, 143–54 (1940)
8. Adlersberg, D., and Sobotka, H., *Gastroenterology*, **1**, 357–65 (1943)
9. Page, R. C., and Bercovitz, Z., *Am. J. Digestive Diseases*, **10**, 174–77 (1943)
10. di Sant 'Agnese, P. A., and Larkin, V. deP., *Proc. Soc. Exptl. Biol. Med.*, **52**, 343–44 (1943)
11. Bolin, D. W., Lampman, C. E., and Berg, L. R., *Poultry Sci.*, **22**, 348–53 (1943)
12. Little, R. W., Thomas, A. W., and Sherman, H. C., *J. Biol. Chem.*, **148**, 441–43 (1943)
13. Rohrer, A. B., and Sherman, H. C., *J. Nutrition*, **25**, 605–9 (1943)
14. Meyer, K. A., Steigmann, F., Popper, H., and Walters, W. H., *Arch. Surg.*, **47**, 26–43 (1943)
15. Nelson, D., Goetzl, S., Robins, S., and Ivy, A. C., *Proc. Soc. Exptl. Biol. Med.*, **52**, 1–2 (1943)
16. Pena, J. G., and Villaverde, M., *Rev. Cubana Cardiologica*, **2**, 332–35 (1940)
17. Grollman, A., and Harrison, T. R., *Proc. Soc. Exptl. Biol. Med.*, **52**, 162–65 (1943)
18. Wakerlin, G. E., and Moss, W. G., *Proc. Soc. Exptl. Biol. Med.*, **53**, 149–52 (1943)
19. Taylor, R. D., and Corcoran, A. C., *Am. J. Med. Sci.*, **206**, 659–67 (1943)
20. Ziskin, D. E., Rosenstein, S. N., and Drucker, L., *Am. J. Ortho. Oral Surg.*, **29**, 163–77 (1943)
21. Remington, R. E., Harris, P. L., and Smith, C. L., *J. Nutrition*, **24**, 597–606 (1942)
22. Mellanby, E., *J. Physiol.*, **101**, 408–31 (1943)
23. Wolbach, S. B., and Bessey, O. A., *Arch. Path.*, **32**, 689–722 (1941)
24. McDermott, W., Webster, B., Baker, R., Lockhart, J., and Tompsett, R., *J. Pharmacol.*, **77**, 24–39 (1943)
25. Sullivan, M., and Evans, V. J., *J. Nutrition*, **25**, 319–39 (1943)
26. Moult, F. H., *Arch. Dermatol. Syphilol.*, **47**, 768–77 (1943)
27. Johnson, M. L., *Arch. Ophthalmol. (Chicago)*, **29**, 793–810 (1943)
28. Irving, J. T., *Dental Record (London)*, **63**, 281–84 (1943)

29. ELDER, J. H., *Science*, **97**, 561–62 (1943)
30. LEGALLEY, D. P., AND HARRISON, J. W. E., *Am. J. Pharm.*, **115**, 95–99 (1943)
31. BRENNER, S., AND ROBERTS, L. J., *Arch. Internal Med.*, **71**, 474–82 (1943)
32. BLANCHARD, E. L., AND HARPER, H. A., *Arch. Internal Med.*, **66**, 661–69 (1940)
33. OLDHAM, H., ROBERTS, L. J., MACLENNAN, K., AND SCHLUTZ, F. W., *J. Pediat.*, **20**, 740–52 (1942)
34. KUHN, H. S., AND WILLE, E. C., JR., *Am. J. Ophthalmol.*, **26**, 63–68 (1943)
35. YUDKIN, J., ROBERTSON, G. W., AND YUDKIN, S., *Lancet*, **245**, 10–13 (1943)
36. LIVINGSTONE, P. C., AND BOLTON, B., *Lancet*, **244**, 263–64 (1943)
37. JEWETT, H. J., SLOAN, L. L., AND STRONG, G. H., *J. Am. Med. Assoc.*, **121**, 566–68 (1943)
38. JOSEPHS, H. W., *Am. J. Diseases Children*, **65**, 712–27 (1943)
39. BODANSKY, O., LEWIS, J. M., AND LILLIENFELD, C. C., *J. Clin. Investigation*, **22**, 643–47 (1943)
40. BYRN, J. N., AND EASTMAN, N. J., *Bull. Johns Hopkins Hosp.*, **73**, 132–37 (1943)
41. STRAUMFJORD, J. V., *Northwest Med.*, **42**, 219–25 (1943)
42. SPECTOR, S., MCKHANN, C. F., AND MESERVE, E. R., *Am. J. Diseases Children*, **66**, 376–95 (1943)
43. LEWIS, J. M., AND HAIG, C., *J. Pediat.*, **15**, 812–23 (1939)
44. BODANSKY, O., LEWIS, J. M., AND HAIG, C., *Science*, **94**, 370–71 (1941)
45. LEWIS, J. M., AND BODANSKY, O., *Proc. Soc. Exptl. Biol. Med.*, **52**, 265–66 (1943)
46. PHILLIPS, P. H., *J. Am. Vet. Med. Assoc.*, **97**, 165–66 (1940)
47. RUBIN, M., AND BIRD, H. R., *Poultry Sci.*, **22**, 53–55 (1943)
48. MATTSON, F. H., AND DEUEL, H. J., JR., *J. Nutrition*, **25**, 103–12 (1943)
49. DEUEL, H. J., JR., HRUBETZ, M. C., MATTSON, F. H., MOREHOUSE, M. G., AND RICHARDSON, A., *J. Nutrition*, **26**, 673–85 (1943)
50. ALMQUIST, H. J., MACKINNEY, G., AND MECCHI, E., *J. Biol. Chem.*, **150**, 99–105 (1943)
51. CREECH, G. T., AND SEIBOLD, H. R., *Am. J. Vet. Research*, **4**, 353–59 (1943)
52. BRAUN, W., AND CARLE, B. N., *J. Nutrition*, **26**, 549–54 (1943)
53. TARASSUK, N. P., AND REGAN, W. M., *J. Dairy Sci.*, **26**, 987–96 (1943)
54. ANDERSON, J. A., HARDENBERGH, J. G., AND WILSON, L. T., *J. Dairy Sci.*, **19**, 483–84 (1936)
55. HOWELL, C. E., HART, G. H., AND ITTNER, N. R., *Am. J. Vet. Research*, **2**, 60–74 (1941)
56. HART, G. H., GOSS, H., AND GUILBERT, H. R., *Am. J. Vet. Research*, **4**, 162–68 (1943)
57. ELLIS, G. H., AND HAMNER, K. C., *J. Nutrition*, **25**, 539–53 (1943)
58. LYON, C. B., BEESON, K. C., AND ELLIS, G. H., *Botan. Gaz.*, **104**, 495–514 (1943)
59. WILSON, R. H., THOMAS, J. O., AND DEEDS, F., *Fruit Products J.*, **22**, 15–17 (1942)
60. ZSCHEILE, F. P., BEADLE, B. W., AND KRAYBILL, H. R., *Food Research*, **8**, 299–313 (1943)

61. MORGAL, P. W., BYERS, L. W., AND MILLER, E. J., *Ind. Eng. Chem.*, **35**, 794–97 (1943)
62. WILLIAMS, K. T., BICKOFF, E., AND VAN SANDT, W., *Science*, **97**, 96–98 (1943)
63. KRUKOVSKY, V. N., *J. Dairy Sci.*, **26**, 869–75 (1943)
64. FUHR, I., DORNBUSH, A. C., AND PETERSON, W. H., *J. Dairy Sci.*, **26**, 643–46 (1943)
65. SOBOTKA, H., KANN, S., AND LOEWENSTEIN, E., *J. Am. Chem. Soc.*, **65**, 1959–61 (1943)
66. FRAPS, G. S., AND MEINKE, W. W., *J. Assoc. Official Agr. Chem.*, **26**, 399–404 (1943)
67. TOMPKINS, P. C., AND BOLOMEY, R. A., *Ind. Eng. Chem., Anal. Ed.*, **15**, 437–38 (1943)
68. COY, N. H., SASSAMAN, H. L., AND BLACK, A., *Ind. Eng. Chem., Anal. Ed.*, **15**, 441–43 (1943)
69. HICKMAN, K., *Ann. Rev. Biochem.*, **12**, 353 (1943)
70. WALL, M. E., AND KELLEY, E. G., *Ind. Eng. Chem., Anal. Ed.*, **15**, 18–20 (1943)
71. HAAGEN-SMIT, A. J., JEFFREYS, E. P., AND KIRCHNER, J. G., *Ind. Eng. Chem., Anal. Ed.*, **15**, 179–80 (1943)
72. PEPKOWITZ, L. P., *J. Biol. Chem.*, **149**, 465–71 (1943)
73. REISER, R., AND FRAPS, G. S., *J. Assoc. Official Agr. Chem.*, **26**, 186–94 (1943)
74. BICKOFF, E., AND WILLIAMS, K. T., *Ind. Eng. Chem., Anal. Ed.*, **15**, 266–68 (1943)
75. JENSEN, J. L., SHANTZ, E. M., EMBREE, N. D., CAWLEY, J. D., AND HARRIS, P. L., *J. Biol. Chem.*, **149**, 473–77 (1943)

VITAMIN D

76. LUND, A. P., AND ARMSTRONG, W. D., *J. Dental Research*, **21**, 513–18 (1942)
77. FUHR, I., AND STEENBOCK, H., *J. Biol. Chem.*, **147**, 59–64 (1943)
78. FUHR, I., AND STEENBOCK, H., *J. Biol. Chem.*, **147**, 65–69 (1943)
79. FUHR, I., AND STEENBOCK, H., *J. Biol. Chem.*, **147**, 71–75 (1943)
80. MCCHESNEY, E. W., *Proc. Soc. Exptl. Biol. Med.*, **52**, 147–49 (1943)
81. CORRELL, J. T., AND WISE, E. C., *J. Nutrition*, **26**, 641–48 (1943)
82. MCCHESNEY, E. W., *J. Nutrition*, **26**, 81–94 (1943)
83. WESSON, L. G., AND BOYLE, P. E., *Arch. Path.*, **36**, 237–42 (1943)
84. BOYLE, P. E., AND WESSON, L. G., *Arch. Path.*, **36**, 243–52 (1943)
85. RICHTER, C. P., AND RICE, K. K., *Am. J. Physiol.*, **139**, 693–99 (1943)
86. SHARPLESS, G. R., SABOL, M., ANTHONY, E. K., AND ARGETSINGER, H. L., *J. Nutrition*, **25**, 119–26 (1943)
87. BRISKIN, H. L., STOKES, F. R., REED, C. I., AND MRAZEK, R. G., *Am. J. Physiol.*, **138**, 385–90 (1943)
88. KATZ, L. N., RODBARD, S., AND MEYER, J., *Am. J. Physiol.*, **140**, 226–29 (1943)
89. MORGAN, A. F., AND SHIMOTORI, N., *J. Biol. Chem.*, **147**, 189–200 (1943)
90. KLASSEN, K. P., AND CURTIS, G. M., *Arch. Internal Med.*, **71**, 78–94 (1943)
91. OPPER, L., AND THALE, T., *Am. J. Physiol.*, **139**, 406–9 (1943)

92. Shimotori, N., and Morgan, A. F., *J. Biol. Chem.*, **147**, 201–10 (1943)
93. Dodds, G. S., and Cameron, H. C., *Am. J. Anat.*, **55**, 135–65 (1943)
94. Dodds, G. S., and Cameron, H. C., *Am. J. Path.*, **14**, 273–96 (1938)
95. Dodds, G. S., and Cameron, H. C., *Am. J. Path.*, **15**, 723–40 (1939)
96. Dodds, G. S., and Cameron, H. C., *Am. J. Path.*, **19**, 169–85 (1943)
97. Ewing, D. T., Kingsley, G. V., Brown, R. A., and Emmett, A. D., *Ind. Eng. Chem., Anal. Ed.*, **15**, 301–5 (1943)
98. Fritz, J. C., and Halloran, H. R., *Poultry Sci.*, **22**, 314–22 (1943)
99. McKay, H., Patton, M. B., Pittman, M. S., Stearns, G., and Edelblute, N., *J. Nutrition*, **26**, 153–59 (1943)

Vitamin E

100. Pappenheimer, A. M., *Physiol. Revs.*, **23**, 37–50 (1943)
101. Heinrich, M. R., and Mattill, H. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 344–46 (1943)
102. Hines, L. R., and Mattill, H. A., *J. Biol. Chem.*, **149**, 549–54 (1943)
103. Dam, H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 285–87 (1943)
104. Kaunitz, H., and Pappenheimer, A. M., *Am. J. Physiol.*, **138**, 328–40 (1943)
105. Hines, H. M., Lazere, B., Thomson, J. D., and Cretzmeyer, C. H., *Am. J. Physiol.*, **139**, 183–87 (1943)
106. Houchin, O. B., and Mattill, H. A., *J. Biol. Chem.*, **146**, 309–12 (1942)
107. Mackenzie, C. G., and McCollum, E. V., *J. Nutrition*, **19**, 345–62 (1940)
108. Weissberger, L. H., and Harris, P. L., *J. Biol. Chem.*, **151**, 543–53 (1943)
109. Evans, H. M., and Emerson, G. A., *J. Nutrition*, **26**, 555–67 (1943)
110. Patrick, H., and Morgan, C. L., *Science*, **98**, 434–35 (1943)
111. Wechsler, I. S., Mayer, G. G., and Sobotka, H., *Proc. Soc. Exptl. Biol. Med.*, **53**, 170–73 (1943)
112. Shute, E., *Urol. Cutaneous Rev.*, **47**, 239–44 (1943)
113. Smith, L. I., and Sprung, J. A., *J. Am. Chem. Soc.*, **65**, 1276–83 (1943)
114. Smith, L. I., and Miller, H. C., *J. Am. Chem. Soc.*, **64**, 440–45 (1942)
115. Robeson, C. D., *J. Am. Chem. Soc.*, **65**, 1660 (1943)
116. Robeson, C. D., and Baxter, J. G., *J. Am. Chem. Soc.*, **65**, 940–43 (1943)
117. Homrich, B. R., *J. Nutrition*, **26**, 391–98 (1943)

Vitamin K

118. Brambel, C. E., and Loker, F. F., *Proc. Soc. Exptl. Biol. Med.*, **53**, 218–20 (1943)
119. Shapiro, S., *J. Lab. Clin. Med.*, **28**, 1596–98 (1943)
120. Page, R. C., and de Beer, E. J., *Am. J. Med. Sci.*, **205**, 257–61 (1943)
121. Stamler, F. W., Tidrick, R. T., and Warner, E. D., *J. Nutrition*, **26**, 95–103 (1943)
122. Maltaner, F., and Maltaner, E., *Arch. Biochem.*, **2**, 37–47 (1943)
123. Day, H. G., Wakim, K. G., Krider, M. M., and O'Banion, E. E., *J. Nutrition*, **26**, 585–600 (1943)
124. Javert, C. T., and Stander, H. J., *Surg. Gynecol. Obstet.*, **76**, 115–22 (1943)
125. Snelling, C. E., *J. Pediat.*, **22**, 77–81 (1943)
126. Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **138**, 21–33 (1941)

127. Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 513–27 (1941)
128. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1–20 (1941)
129. Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, **23**, 589–602 (1942)
130. Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*, **147**, 463–74 (1943)
131. Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 529–34 (1941)
132. Shapiro, S., Redish, M. H., and Campbell, H. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 12–15 (1943)
133. Davidson, C. S., and MacDonald, H., *Am. J. Med. Sci.*, **205**, 24–33 (1943)
134. Davidson, C. S., and MacDonald, H., *New Engl. J. Med.*, **229**, 353–55 (1943)
135. Meyer, O. O., and Howard B., *Proc. Soc. Exptl. Biol. Med.*, **53**, 234–37 (1943)
136. Shapiro, S., Redish, M. H., and Campbell, H. A., *Proc. Soc. Exptl. Biol. Med.*, **53**, 251–54 (1943)
137. Shemiakin, M. M., Schukina, L. A., and Shvezov, J. B., *Nature*, **151**, 585–86 (1943)
138. Dam, H., *Nature*, **152**, 355 (1943)
139. Ewing, D. T., Tomkins, F. S., and Kamm, O., *J. Biol. Chem.*, **147**, 233–41 (1943)
140. Morton, C. B., Sheaburn, E. W., and Burger, R. E., *Surgery*, **14**, 915–20 (1943)
141. Maltaner, F., and Thompson, W. R., *Arch. Biochem.*, **2**, 49–54 (1943)
142. Vivino, A. E., Haydak, M. H., Palmer, L. S., and Tanquary, M. C., *Proc. Soc. Exptl. Biol. Med.*, **53**, 9–11 (1943)

New Factor

143. Wulzen, R., and Bahrs, A. M., *Physiol. Zoöl.*, **8**, 457–73 (1935)
144. Bahrs, A. M., and Wulzen, R., *Proc. Soc. Exptl. Biol. Med.*, **33**, 528–32 (1936)
145. Wulzen, R., and Bahrs, A. M., *Physiol. Zoöl.*, **9**, 508–29 (1936)
146. Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, **133**, 500 (1941)
147. van Wagtendonk, W. J., and Wulzen, R., *Arch. Biochem.*, **1**, 373–77 (1942–43)
148. Kohler, G. O., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **15**, 445–59 (1938)
149. Gouley, R. M., *Master's Thesis* (Oregon State College, Corvallis, Oregon, 1941)

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NUTRITION

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From the standpoint of the relation of nutrition to human welfare one of the most important activities ever instituted was begun by the United Nations Conference on Food and Agriculture at Hot Springs, Virginia, in May 1943 (1). This conference at which forty-four nations were represented accepted the principle that governments individually and collectively must assume the responsibility for seeing that the people of the world have an opportunity to obtain a food supply adequate for health, and that agricultural policies should be directed toward this end. The conference consisted of three sections. Section I dealt with consumption levels and requirements; Section II considered expansion of production and adaptation of consumption needs; and Section III dealt with facilitation and improvement of distribution.

The deliberations of Section I are of especial interest to nutritionists. It was pointed out that the diet of large sections of the population in all countries does not attain the level needed for health and that a vast increase in food production in every country will be necessary to provide the food needs of mankind. The table of "Recommended Dietary Allowances" of the National Research Council (2) was recognized as an example of a yardstick which can be applied, for limited groups, on a family basis, on a national basis, or to the world as a whole. This table was reproduced in part in the *Annual Review of Biochemistry* (2a). For the sake of completeness and ease of reference, it is given here (see pages 442–43) in its complete form including recommendations which were adopted in 1942.

In order to make practical use of the "Recommended Dietary Allowances" it is necessary to translate them into terms of food commodities. Although the nutritive requirements may have world-wide application a set of food commodities has limited application, and innumerable combinations of individual foods may be devised to form a nutritionally adequate food supply to meet varying tastes and re-

TABLE I

RECOMMENDED DIETARY ALLOWANCES*

FOOD AND NUTRITION BOARD, NATIONAL RESEARCH COUNCIL

	Calories	Protein grams	Calcium grams	Iron mg.	Vitamin A‡ I.U.	Thiamin (B_1) mg.†	Riboflavin mg.	Niacin (Nicotinic acid) mg.	Ascorbic acid mg.†	Vitamin D I.U.
Man (70 Kg.)										
Sedentary	2500	....	......	......	......	1.5	2.2	15	......	....
Moderately active	3000	70	0.8	12	5000	1.8	2.7	18	75	**
Very active	4500	....	......	......	......	2.3	3.3	23	......	....
Woman (56 Kg.)										
Sedentary	2100	....	......	......	......	1.2	1.8	12	......	....
Moderately active	2500	60	0.8	12	5000	1.5	2.2	15	70	**
Very active	3000	....	......	......	......	1.8	2.7	18	......	....
Pregnancy (latter half)	2500	85	1.5	15	6000	1.8	2.5	18	100	400 to 800
Lactation	3000	100	2.0	15	8000	2.3	3.0	23	150	400 to 800
Children up to 12 years:										
Under 1 year§	100/Kg.	3 to 4/Kg.	1.0	6	1500	0.4	0.6	4	30	400 to 800
1–3 years¶	1200	40	1.0	7	2000	0.6	0.9	6	35	**
4–6 years	1600	50	1.0	8	2500	0.8	1.2	8	50	....
7–9 years	2000	60	1.0	10	3500	1.0	1.5	10	60	....
10–12 years	2500	70	1.2	12	4500	1.2	1.8	12	75	....
Children over 12 years:										
Girls, 13–15 years	2800	80	1.3	15	5000	1.4	2.0	14	80	**
16–20 years	2400	75	1.0	15	5000	1.2	1.8	12	80	....
Boys, 13–15 years	3200	85	1.4	15	5000	1.6	2.4	16	90	**
16–20 years	3800	100	1.4	15	6000	2.0	3.0	20	100	....

* Tentative goal toward which to aim in planning practical dietaries; can be met by a good diet of natural foods. Such a diet will also provide

Boys, 13–15 years................	3200	85	1.4	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16–20 years................	3800	100	1.4	15	6000	2.0	3.0	20	100	....

* Tentative goal toward which to aim in planning practical dietaries; can be met by a good diet of natural foods. Such a diet will also provide other minerals and vitamins, the requirements for which are less well known.

† 1 mg. thiamin equals 333 I.U.; 1 mg. ascorbic acid equals 20 I.U.

‡ Requirements may be less if provided as vitamin A; greater if provided chiefly as the pro-vitamin carotene.

§ Needs of infants increase from month to month. The amounts given are for approximately 6–8 months. The amounts of protein and calcium needed are less if derived from human milk.

¶ Allowances are based on needs for the middle year in each group (as 2, 5, 8, etc.) and for moderate activity.

** Vitamin D is undoubtedly necessary for older children and adults. When not available from sunshine, it should be provided probably up to the minimum amounts recommended for infants.

Further Recommendations, Adopted 1942:

The requirement for *iodine* is small; probably about 0.002 to 0.004 milligram a day for each kilogram of bodyweight. This amounts to about 0.15 to 0.30 milligram daily for the adult. This need is easily met by the regular use of iodized salt; its use is especially important in adolescence and pregnancy.

The requirement for *copper* for adults is in the neighborhood of 1.0 to 2.0 milligrams a day. Infants and children require approximately 0.05 per kilogram of bodyweight. The requirement for copper is approximately one-tenth of that for iron.

The requirement for *vitamin K* is usually satisfied by any good diet. Special consideration needs to be given to newborn infants. Physicians commonly give vitamin K either to the mother before delivery or to the infant immediately after birth.

sources. The foods of the world were grouped into the broad categories of

I. Grain products
II. Vegetables and fruits
   a. Starch-rich roots, tubers, or fruits
   b. Leguminous seeds
   c. Leafy, green, yellow vegetables
   d. Vitamin-C–rich fruits
   e. Other vegetables or fruits
III. Milk fluid, powdered or evaporated, cheese, or in various other forms
IV. Meats, fish, poultry, and eggs
V. Fats and oils
VI. Sugar, syrups, molasses, honey, and sweet preserves
VII. Beverages; condiments, sauces, salts

The Conference gave two examples of the many possible food groupings based on the table of "Recommended Allowances" which are adequate provided reasonably good nutritional choices are made within each food group (Table II). Plan I is meant to be relatively economical, with plan II relatively costly.

TABLE II

KILOGRAMS PER PERSON PER YEAR

Food Groups	Plan I	Plan II
Grain products	104	88
Milk (as fluid)	217	240 (liters)
Starch-rich tubers, roots, fruits	82	68
Mature leguminous seeds and nuts	11	6
Vitamin-C-rich fruits	36	43
Leafy, green, yellow vegetables	36	70
Other vegetables and fruits	54	88
Meats, fish, poultry	41	54
Eggs	228	276 (numbers)
Sugars	16	16
Fats	23	23

It was recognized that in practice it is often necessary to adjust dietary recommendations to existing economic and agricultural realities and that the task of nutrition experts is to make recommendations for improved agricultural and economic policies in accordance with

the recommended allowances. In the poorer countries of the world it is not enough to recommend diets which are just sufficiently satisfactory to prevent serious malnutrition. While this may be expedient at times, improved diets above such unsatisfactory levels should be insisted on in all possible circumstances. Aspects of nutrition education were discussed, public health and sociological aspects were considered. Boudreau (3) in discussing the effects of the recommendations on this country points out that one of the first things that must be done is to establish a national nutrition organization if one does not now exist to guide food and nutrition policy but not to administer it. According to the Conference recommendations this national nutrition organization will among other things exchange information and experiences with other national nutrition organizations, send representatives to meet regularly with representatives of other national nutrition organizations, report on results of dietary and nutrition studies, and on the success in raising the national level of food consumption. Boudreau states that "The conference painted a picture of how common men and women would profit in diet and health in a post-war world in which nations would act together for the welfare of all." Parran (4) has presented the results of the conference from the health point of view. He calls attention to the reports presented from every continent pointing to a world-wide under-consumption of food leading to malnutrition and deficiency disease. Nutrition links public health with agriculture and because medical and health administrators can contribute a knowledge of the existence of malnutrition and its elimination they have a great responsibility in the field of nutrition. Parran states that "any advance in health through better nutrition will demand the full collaboration of public health and agricultural authorities." The Conference set up an interim commission to function until a permanent commission is set up. Thus a continuing organization should insure that the results of the conference will actually be translated into international action. If the agreeing nations will now adequately implement the continuing commission the first step has been taken toward accomplishing one of the greatest advances in health and agriculture that the world has ever seen.

Nutrition, Public Health, and Industry

During the year there has been a growing consciousness of the important relation between health and nutrition. The world-wide distribution of the deficiency diseases was indicated in a review of the

available medical reports by Sebrell (5, 6) who also points out some of the public health aspects of nutrition. Additional dietary survey work has continued to indicate that large numbers of people in this country have a nutritional status below the National Research Council's recommended allowances. Youmans, Patton & Kern (7) in one of a series of papers covering extensive studies of a large population group in Tennessee found a serious deficiency in calories on the basis of the recommended allowances, accompanied by a significant and often severe deficiency in mean body weight although all subjects were not abnormally underweight. They suggest that the deficiency in calories is not as great as it appears and that this may be due in part to the recommended standards being too high. Youmans, Patton *et al.* (8) in a later paper on the protein status found frequent and severe deficiencies in intake and a significant incidence of protein deficiency as manifested by a hypoalbuminemia. The remainder of the papers in this series should be of equally great interest to students of nutrition. Phipard (9) points out, however, that the data show a general dietary improvement in the United States for 1942 over 1936, and Stiebeling (10) in a review of the adequacy of American diets gives the per capita consumption of various types of foods in this country over a period of about forty years and compares the dietary situation in the early 1940's with that of the middle 1930's. For economic reasons it seems possible that at least one family in every seven that had poor diets in 1936 was able to obtain a fair or good diet in 1941. Activities such as the food stamp program, direct food distribution, government aided milk and school lunch programs, as well as increased public interest and education in nutrition, all tended to improve diets. Stiebeling points out that for 1942 if the agricultural goals were obtained and no unforeseen demands beyond those of early 1942 developed the per capita consumption of domestically produced foods can be maintained at a level as high as or higher than in the recent past.

Manning & Milam (11) in a survey of youths in North Carolina under the National Youth Administration found 85 per cent of the boys admitted in a twelve-month period had unsatisfactory levels of plasma ascorbic acid (below 0.6 mg. per cent). Only 12 per cent showed low plasma levels of vitamin A (less than 70 I.U. per 100 cc.). Nicotinic acid saturation tests carried out on 53 youths gave a value between the levels of a group of normal adequately nourished individuals and another of unselected hospital admissions.

A nutrition survey in East York Township, Canada, by Riggs, Perry *et al.* (12) is of particular interest because it points out the doubtful value of several specific tests and the urgent need for the development of reliable methods for nutritional examinations, particularly for the subclinical states. This survey was made on 546 students in the East York Collegiate Institute. An evaluation of food consumption showed a low consumption of foods containing thiamin and ascorbic acid. Although 75 per cent of the girls and 80 per cent of the boys had calculated intakes of less than 50 mg. of ascorbic acid daily there were no cases of marked gingivitis or other evidence of ascorbic acid deficiency. Dental conditions were unsatisfactory. No symptoms that could be attributed to thiamin deficiency were observed. The nutritional status, as judged by a thorough physical examination, and the health of most of the students were good. Attempts to correlate the findings were unsatisfactory and the authors conclude that the assessment of nutritional status in a large group is at present difficult, if not impossible, when definite clinical signs of deficiencies are absent.

The data in the above studies make clear the need for better methods of early recognition of the mild symptoms of these conditions and the increasing need for the use of medical and public health personnel in determining the real prevalence of deficiency diseases.

Winters & Leslie (13) in a study of a small group of low income women in Texas found severe deficiency in intake of calories, protein, minerals, and vitamins. They also found a fairly close correlation between the analytical data and the finding of physical evidence of deficiency, with the exception of thiamin. No severe cases of deficiency were found and these authors also suggest the possibility that the "Recommended Dietary Allowances" for the sedentary women have been placed too high. Hardy, Spohn *et al.* (14) in a study of more than 7,000 children in Chicago found 60 per cent with physical manifestations suggestive of poor nutrition, while 67 per cent of the white children and 89 per cent of the negroes failed to meet a minimum adequate dietary standard. Kelly & Sheppard (15) from dietary and questionnaire data in a group of about 450 adults of upper income level found 76 per cent deficient in thiamin and 77 per cent in riboflavin. Harris, Weeks & Kinde (16) studying 760 children between the ages of nine and twelve living in small Michigan communities found on a conservative basis that 50 per cent had low plasma ascorbic acid, 50 per cent had avitaminosis A by biomicroscope, 53 per cent

showed ariboflavinosis by biomicroscope, and 39.7 per cent had low hemoglobin values. The addition of an especially prepared dehydrated soup to the diet of 426 children for three months showed a significantly greater improvement in ascorbic acid and iron status, no significant improvement in weight and riboflavin status, and no change in height or vitamin A status as compared with a control group.

Sebrell & Wilkins (17) have stressed the need for closer co-operation between health authorities and nutrition workers and indicated the role health departments can play in such activities. Kendrick (18) has described the operation of such a program in North Carolina and Amyot (19) reported on the operation of nutrition programs in Canada.

The interest in nutrition programs naturally has led to more attention to the possible effects of malnutrition on physical fitness, health, and production of industrial workers. A review of the subject of physical performance in relation to diet with more than 400 references was published by Keys (20) who points out that although there is little question that physical performance is hindered when real deficiencies exist the real problem is what can be done by dietary measures to improve the performance of persons who are not clearly malnourished. The literature although voluminous does not furnish a satisfactory answer and work done during 1943 still does not solve the problem. Harper, Mackay *et al.* (21) in a study of sixty-nine British university students, comparing the effects of supplements of vitamins A, C, and D with controls, found a greater increase in resting vital capacity, in breath holding, and in endurance times measured by ability to maintain a column of mercury at 40 mm. by steady expiration in the vitamin supplemented group. This group also had a faster resting pulse rate.

Jenkins & Yudkin (22) studying 178 children, about half of whom were given vitamin supplements every school day for one year, did not find any difference in resting pulse rate, vital capacity, breath holding, and endurance tests from the half receiving control pellets. They conclude that the reason no difference was seen may have been that the children were not initially deficient in the supplementary nutrients.

In a study of the possible effects of thiamin on performance, Keys, Henschel *et al.* (23) were unable to demonstrate any benefit in healthy young men on about 3,000 calories per day from an intake of more than 0.23 mg. of thiamin per 1,000 calories over a period of about three months. Williams, Mason *et al.* (24) observed severe weakness and

anorexia in two volunteers on a thiamin intake restricted to 0.1 mg. per 1,000 calories. Polyneuropathy became clearly apparent after 110 days. Barborka, Foltz & Ivy (25) studying four medical students on a vitamin B complex deficient diet, using a bicycle ergometer, observed fatigue, anorexia, and other subjective symptoms (without any objective evidence of deficiency disease) with a decreased work output which was restored to efficient levels by vitamin B complex supplementation. The thiamin intake was 0.65 mg. and the riboflavin intake 0.94 mg. A possible explanation for some of the apparent discrepancies in former work with thiamin was suggested in an important paper by Najjar & Holt (26). Nine adolescent male volunteers were given gradually reduced thiamin intakes over a period of many months. Eventually after an intake of 0.1 to 0.2 mg. per day for months thiamin was omitted entirely from the diet. Four of the nine subjects then showed no signs of deficiency during a seven weeks' observation period. In spite of the demonstration that the diet contained no thiamin, each of these subjects had large quantities of free thiamin in the feces. The authors show that the intestinal bacteria were the source of this thiamin. This work in addition to offering a possible explanation for variations in clinical results on different dietary intakes of thiamin also opens up a new field of investigation, namely, a study of the effect of diet composition on intestinal bacterial vitamin synthesis and the role of this synthesis in meeting human vitamin requirements.

The possibility of dietary inadequacy adversely affecting work output in industry and the possibility that food as served in industrial plants may have undergone important nutritive losses in preparation have led to considerable investigation in these fields. Wiehl (27) observed in a survey of the diets of more than a thousand workers in a large aircraft factory that more than 87 per cent of the diets studied fell below the National Research Council's Recommended Allowances. Borsook, Alpert & Keighley (28) studying 1,173 aircraft workers found, according to the criteria used, nearly all with evidence of vitamin A deficiency; 17 to 26 per cent with thiamin deficiency; 9 per cent with oral and 35 per cent with ocular lesions associated with riboflavin deficiency; 2.1 per cent with severe niacin deficiency; 32 per cent with plasma ascorbic acid of less than 0.4 mg. per cent, and approximately 11 per cent were anemic. Goodhart (29) in a study of food as served found that as much as 90 per cent of the thiamin present in the fresh raw food was lost before the food was eaten. Stiebeling (10) has indicated that estimates of dietary adequacy may

give an overly optimistic result unless the losses in preparation and cooking of foods are taken into consideration. In a study of the effect of cooking on the vitamin retention in fresh pork McIntire, Schweigert *et al.* (30) found losses in thiamin of from 30 to 50 per cent in the meat itself, depending on the method of cooking; nicotinic acid losses were from 15 to 35 per cent, and riboflavin 15 per cent. In a further study of fresh, fresh-stored, and cured hams, Schweigert, McIntire & Elvehjem (31) found an average retention in the meat alone after roasting of 58 per cent of the thiamin, 79 per cent of the nicotinic acid, and 75 per cent of the riboflavin. Ten to 15 per cent of each was found in the drippings from roasting and frying. A higher retention of thiamin in the meat was found after frying as compared with roasting, braising, and broiling. A portion of the losses was found in the drippings. The nicotinic acid content of a number of vegetables, legumes, and fruits, using the microbiological assay method, was determined by Russell, Taylor & Beuk (32). The liquid associated with the canned vegetables contained from 30 to 40 per cent of the total nicotinic acid. During cooking, losses varied from 8 per cent in fresh legumes, to 22 per cent in leafy plants. The cooking water contained from 2 to 41 per cent of the total nicotinic acid. Oser, Melnick & Oser (33) observed the losses in five vitamins and three minerals from peas, potatoes, carrots, and broccoli with two different methods of cooking. Variable and in some instances very large losses of vitamins (averaging 31 per cent) occurred. The cooking method using minimal amounts of water and minimal loss of steam resulted in the greatest conservation. Cheldelin, Woods & Williams (34) observed losses, due to cooking, of riboflavin, nicotinic acid, pantothenic acid, biotin, inositol, and folic acid in thirty foods. Microbiological assay methods were used. Variable losses from negligible to moderate were found except for folic acid. Losses of folic acid were very great for most foods. Heller, McCay & Lyon (35) determined the losses in vegetables in a large industrial cafeteria under actual operating conditions. They found losses in thiamin of 16 to 64 per cent, niacin 2 to 61 per cent, riboflavin 22 to 45 per cent, and ascorbic acid 27 to 90 per cent. These same workers (36) found that the noon meal in one cafeteria in a Navy Yard furnished at most only one-fourth of the day's requirements for riboflavin, niacin, and thiamin. Dry brewers' yeast was used as a method of supplementation.

Bing (37) is of the opinion that additional properly planned experiments must be run by competent investigators to determine the im-

portance of nutrition in industrial production. He points to the importance of improved lunchroom facilities, properly prepared foods, and the use of educational material. Cowgill (38) has presented some of the important aspects of industrial nutrition and cites some of the activities now being carried out. It is obvious that the proper feeding of industrial workers is a problem of great importance especially with a controlled food distribution. It is also clear that although there are still not enough data to show precisely the effects of slight degrees of malnutrition on industrial production or the extent of malnutrition among industrial workers in this country, the indications to date are that the problem is one of great magnitude. Therefore, it has been necessary to institute a national nutrition program for industry. Goodhart (39) has reviewed the mechanism of this program which already is underway. This program is designed now to cover the furnishing of adequate food, equipment, and manpower to war industries for a good food service, to improve methods of preparation and serving so that nutrients will not be lost in unduly large amounts, and to furnish nutrition educational material designed to improve food selection.

The possible loss of vitamins in sweat as a factor in industrial nutrition and vitamin requirements under tropical conditions led to a study of this subject. Tennent & Silber (40) determined the excretion of ascorbic acid, thiamin, riboflavin, and pantothenic acid in both thermal sweat and sweat induced by exercise. No ascorbic acid was found and the average loss of dehydroascorbic acid was 0.23 mg. Insignificant amounts of thiamin were excreted. The average excretion of riboflavin was 10 μg. per hour and of pantothenic acid 25 μg. per hour. Losses of thiamin, riboflavin, and pantothenic acid were not greatly influenced by dosing the subjects with vitamins. Similar results were obtained by Cornbleet, Kirch & Bergeim (41) who determined thiamin, riboflavin, niacin, and pantothenic acid in thermal sweat. They found values per liter of sweat of thiamin 150 μg., of riboflavin 120 μg., of pantothenic acid 300 μg. and of nicotinic acid 200 μg. They note that these values may be high for sweat as excreted because of concentration due to evaporation on the skin. The administration of the vitamins did not lead to any noticeable increase in secretion in the sweat. Mickelsen & Keys (41a) also found negligible losses of thiamin, riboflavin, ascorbic acid, and nicotinic acid in sweat. The values are so low that the losses of these substances by this route are of little or no practical importance unless sweating is very profuse or the diet low in these vitamins.

Vitamins and Sulfonamide Drugs

Several important contributions in this field appeared in 1942. It was shown that various sulfa drugs produced vitamin deficiencies in experimental animals on purified diets and efforts were made to determine whether the observed effects were due to interference with bacterial vitamin synthesis in the intestine. The active interest in these relationships continued during 1943. Gant, Ransome *et al.* (42) observed a decrease in *E. coli* in the feces and cecum of rats receiving sulfaguanidine and succinylsulfathiazole. Supplementation with liver extract or a crude "folic acid" concentrate resulted in maintenance of normal growth of the animals. Lewis, Ham & Jensen (43) found the death rate in chickens on an adequate ration with sulfaguanidine to be less than half that occurring when a purified ration was used and a mixture of *p*-aminobenzoic acid, thiamin, and riboflavin or a suspension of *E. coli* were administered with the drug. Newmann, Krider & Day (44) confirmed the previous observation of Daft, Ashburn & Sebrell (45) that rats on purified diets containing succinylsulfathiazole developed biotin deficiency. West, Jefferson & Rivera (46) showed that pantothenic acid deficiency occurred in rats on a purified low casein diet containing sulfapyridine. The deficiency was reversible by the addition of pantothenic acid. They also state that retardation of growth under these conditions could be partially corrected by either *l*-cystine or *dl*-methionine. Daft, Endicott *et al.* (47) produced vitamin E deficiency in rats on purified diets with succinylsulfathiazole.

In 1942 it had been shown by Spicer, Daft *et al.* (48) that rats on purified diets given sulfanilylguanidine or succinylsulfathiazole developed granulocytopenia and leukopenia which could be prevented or successfully treated by dried liver or certain liver extracts. This finding was confirmed by Axelrod, Gross *et al.* (49). Kornberg, Daft & Sebrell (50) also observed severe granulocytopenia and anemia in rats on purified diets fed sulfathiazole, sulfadiazine, or sulfanilamide. These blood dyscrasias also were corrected by liver extracts and late in the year Daft & Sebrell (51) reported the successful treatment of the granulocytopenia and leukopenia with solutions of crystalline "folic acid." Totter & Day (52) had previously stated that xanthopterin was effective in alleviating leukopenia in rats given succinylsulfathiazole. Others however were unable to confirm this observation (49, 51). Totter, Shukers *et al.* (53) also observed leukopenia and anemia in monkeys, with restoration of hemocytopoietic function in an animal

with heated liver and xanthopterin. They suggest that xanthopterin is necessary for the monkey but that unidentified substances are also necessary to prevent vitamin M deficiency. Wright & Welch (54) working with incubated liver slices observed an increase in the formation of "folic acid" by the addition of synthetic xanthopterin and suggest that xanthopterin or a substance derived from it may constitute a portion of the folic acid molecule.

O'Dell & Hogan (55) working with a previously observed anemia in chicks found that sulfaguanidine increased the incidence of the anemia which responded to a beef liver fraction containing a substance designated as vitamin B_c. Pfiffner, Binkley *et al.* (56) reported the isolation of this substance from liver and the formation of the methyl ester which can be converted back to the crystalline acid. They also state that the substance is probably identical with Peterson's eluate factor and Williams' folic acid. Stokstad (57) reported the isolation of a substance from liver which appears to be identical with that of Pfiffner *et al.* They also isolated a material apparently closely related to it from yeast which had only half the growth activity for *S. lactis* R. The isolation of another bacterial growth factor was also reported by Keresztesy, Rickes & Stokes (58). This substance differs from "folic acid" in that although it will replace "folic acid" as a growth factor for *S. lactis* R., it is inactive for *L. casei* E. The possible chemical and biological interrelationship of these substances remains to be determined. The terminology is at present confused and unsatisfactory; this should be clarified when further information on the chemical structure is available.

Interesting data in the field of vitamin interrelations were presented by Wright & Welch (59) who found that the addition of succinylsulfathiazole to purified diets containing pantothenic acid results in changes interpreted by them to be evidence of pantothenic acid deficiency accompanied by reduction in the pantothenic acid content of the liver. These changes, however, were not corrected by pantothenic acid but were corrected by including crystalline biotin and "folic acid" concentrates in the diet. Welch & Wright (60) also found inhibition of growth and increased prothrombin time, and related the mechanism to the effect of succinylsulfathiazole on bacterial activity in the intestine. Working with the nutritional requirements of monkeys, Saslaw, Wilson *et al.* (61) reported a leukopenia which responded to administration of a yeast residue containing "folic acid" together with unidentified substances. Waisman, Rasmussen *et al.* (62) also

working with monkeys observed leukopenia and anemia among other changes. The normal blood picture was maintained by liver or certain liver extracts. Waisman & Elvehjem (63) then showed that the leukopenia was quickly alleviated by the administration of an impure "folic acid" preparation.

All of the above work indicates that our knowledge is incomplete and the field is still a fertile one for research. Lesions in the arteries, heart, and liver of unexplained etiology have been reported by Ashburn, Daft *et al.* (64). The widespread use of drugs which may affect intestinal bacteria or body metabolism and which also may produce a variety of toxic effects offers a field of possible practical application which may be of the greatest importance. Holmes (65) has reported an increased excretion of ascorbic acid in patients given sulfathiazole.

Nutrition of Bacteria and Protozoa

The nutrition of bacteria has attracted special attention. The amazing effect of minute amounts of essential nutrients on bacterial growth had led to work on the nutritional requirements of bacteria and the use of the growth of organisms on synthetic media as a means of assay for the constituents of the media.

Berkman (66) cultivated members of the genus *Pasteurella* on a hydrolyzed gelatin basal media by the addition of nicotinamide and pantothenic acid. They also found that nicotinic acid could not substitute for the amide. McCullough & Dick (67) showed that certain strains of *Brucella* grown on synthetic media require certain concentrations of thiamin, nicotinic acid, calcium pantothenate, and biotin. Johnson & Rettger (68) compared the nutritional requirements of *Salmonella typhosa, S. pullorum,* and *S. gallinarum* and found that only strains of *S. gallinarum* required the addition of thiamin. Underkofler, Bantz & Peterson (69) found that *A. suboxydans* requires pantothenic acid, *p*-aminobenzoic acid, and nicotinic acid but synthesizes riboflavin and biotin. Bovarnick (70) reported that heating asparagine and glutamic acid together in neutral solution at 100°C. for several days forms a substance which can replace nicotinamide in promoting the growth of *B. dysenteriae, Staphylococcus aureus,* and *L. arabinosus.* She also showed (71) that biological assay with *L. arabinosus* and chemical assay by cyanogen bromide and aniline indicate that the substance formed probably is nicotinamide. In a later paper (72) it was demonstrated that the reaction is promoted by aeration and accelerated by manganese and iron salts. The ammonium

salts of a few dibasic acids (aspartic, α-ketoglutaric, maleic, and malic) when heated with glutamic acid produced slight nicotinamide activity although the sodium salts were completely inactive. A number of amino acids and non-nitrogenous dibasic acids (methionine, proline, glutaric acid, etc.) were also found to be capable of substituting for glutamic acid in the reaction although all were less active with the exception of methionine. The only effective substitute for asparagine yet found is glutamine.

Landy, Larkum & Oswald (73) demonstrated that although several organisms require *p*-aminobenzoic acid for growth a number of genera grown on media free of *p*-aminobenzoic acid are able to synthesize this substance, this fact indicating that it is an essential metabolite for bacteria. Landy, Larkum *et al.* (74) also showed that strains of *S. aureus* which had been made sulfathiazole-resistant produced 70 times as much *p*-aminobenzoic acid as the non-resistant parent strains. Tepley, Axelrod & Elvehjem (75) found an acid labile material in grass juice and a liver extract distinct from *p*-aminobenzoic acid which counteracts sulfapyridine bacteriostasis of *L. arabinosus*. Properties of this material were similar to those reported for "folic acid" concentrates. Dorfman & Koser (76) observed that sulfapyridine and sulfathiazole in contrast to sulfanilamide would inhibit the nicotinamide stimulated respiration of *B. dysenteriae* grown on a medium deficient in nicotinamide. This inhibition could not be reversed by *p*-aminobenzoic acid.

Interest in the nutrition of protozoa has been stimulated because of the possible relationship to the development of the malaria parasite. The nutrition of protozoa was the subject of an extensive review by Doyle (77) who points out that our knowledge of the metabolism of representative protozoa is still fragmentary. Notable species and strain variations in requirements for fatty acid metabolism, nitrogen, plant hormones, and vitamins have been found. The nutritional requirements of these forms appear to be very complex and numerous specific nutrients are required. Since the phylum contains types which vary from chlorophyll-bearing plant-like forms to those which seem to require living particulate food, wide variations in nutritive requirements are to be expected. Doyle points out that these specialized requirements have demonstrated interesting evolutionary relationships. A detailed review of the growth factors for protozoa by Hall (78) indicates our lack of knowledge concerning their nutritive requirements. There is no conclusive evidence that protozoa synthesize vitamins although this

has been taken for granted in the case of the plant-like flagellates. Hall tabulates the data both on the thiamin requirements of the plant-like flagellates and the vitamin and other growth factors required by the higher protozoa. He also discusses the interesting field of unidentified growth factors essential in protozoan metabolism. Peterson (79) has observed four growth factors necessary for the ciliate protozoan colpidium which have properties different from those of the well-known vitamins. He has designated these factor B, factor F_1, factor F_2, and factor IV. All of these factors are contained in yeast. No growth was obtained using proteose-peptone which contained riboflavin, pantothenic acid, and thiamin. Garnjobst, Tatum & Taylor (80) found that *Colopoda duodenaria* requires the intact pantothenic acid molecule, riboflavin, thiamin, pyridoxine, and nicotinamide as well as unidentified heat-stable growth factors.

Trager (81) studying avian malaria showed that *P. lophurae* in ducks remained at a high level longer, and more died among the biotin deficient birds than among the controls. Chickens with slight biotin inadequacy also developed more severe infections than those given more nearly adequate amounts of biotin. Biotin deficient ducks infected with *P. cathemerium* had infections that persisted for several days after the blood of the controls was virtually free of demonstrable parasites. Several of the biotin deficient ducks died from the infection. In ducks infected with *P. lophurae* there was an increase in the biotin level in both plasma and red blood cells which returned to normal by the eighth day after inoculation. Trager (82) in further studies on the malaria parasite *P. lophurae* succeeded in obtaining survival of the parasite *in vitro* for about two weeks in a medium to which calcium pantothenate had been added. In the absence of added pantothenate no exflagellants were seen after the fifth day, but added pantothenate exflagellants persisted for twelve to sixteen days. No consistently favorable result was observed with biotin or a mixture of thiamin, pyridoxine, nicotinic acid, and riboflavin.

Nutrition, Reproduction, and Lactation

There have been numerous studies designed to yield information on the effect of nutritional status on fertility, mother, and offspring. Although considerable progress has been made there is still need for much additional work in order to determine the exact nutritive factors involved in obtaining maximum fertility, best status of the mother, and healthiest offspring. Foster, Jones *et al.* (83) working with mice

raised a colony to the fourth generation on highly purified diets in which all the vitamins, essential fatty acids, and salts were supplied as chemically pure compounds. Fertility was good but growth was subnormal and the mortality rate was higher than stock animals. They conclude that the diet is inadequate in some respect and that the addition of 2 per cent of a liver extract would not completely correct it. Taylor, Pennington & Thacker (84) found that the addition of calcium pantothenate to a commercial dog chow fed to rats and mice increased the average litter size in a group of 60 litters by 24 per cent over a group of 58 control litters. The brain and heart weights of day-old rats were relatively less in the pantothenate supplemented group. The great importance of calcium was indicated in a study by Boelter & Greenberg (85). These workers found that rats reared from weaning on a diet containing about 10 mg. of calcium per 100 gm. of food failed to mate and mothers transferred to a low calcium diet showed a marked decrease in fertility. Pregnancy did not cause a great drain on the calcium stores of the mother but lactation reduced the skeletal calcium. Young that were able to survive were almost normal at birth except for low bone calcium.

One of the most interesting developments has been the work on the effect of inadequate nutrition on congenital malformations. Warkany, Nelson & Schraffenberger (86) in a continuation of previous studies showed that congenital malformations in rats fed a deficient diet could be prevented by the addition of liver or an alcoholic liver extract. The addition of liver after the fourteenth day of gestation would not afford complete protection, and it is concluded that the developmental period between the thirteenth and fourteenth day is the critical period in which the nutritional factor exerts its influence. These same authors in a further study of the nature of the congenital malformations (87) observed that the defects of ossification producing syndactylism, shortening and curving of the tibia, fibula, ulna, and radius, feet pointing backward and inward, and other changes are secondary results due to defects which date back to the cartilaginous or precartilaginous stage of the structures affected. Warkany & Schraffenberger (88) found that these changes occurred when a synthetic diet deficient in riboflavin was used and Warkany (89) reported that the defects failed to appear when riboflavin was included in the diet. It thus appears that riboflavin through some effect at the cartilaginous or precartilaginous stage of skeletal development is the essential factor involved in these congenital malformations.

The extent to which nutrition may affect the course of human pregnancy and infant well-being has not yet received the full investigation which the subject deserves. Ebbs (90) has reviewed the nutritive requirements in pregnancy and lactation. He points out the increased requirements for calories, minerals, vitamins, and protein. Women on a good diet have fewer miscarriages, premature births, stillbirths, and infections, and the incidence of illness among the babies is less than is the case when a poor diet is taken. A study of the relation of nutritional inadequacy to anemia in pregnancy was made by Bethell, Blecha & Van Sant (91). Twenty-five per cent of 484 pregnant women in rural Michigan were found to be anemic. Both hypochromic and macrocytic anemia were observed. The hypochromic anemia was associated with the iron intake. Approximately 35 per cent of the hypochromic anemia patients had an average daily iron intake of less than 8 mg. The most interesting finding was the association between protein intake and macrocytic anemia. Only about 2 per cent had macrocytic anemia when the average daily protein intake was over 79 gm., but the incidence was about 27 per cent in those getting less than 40 gm. of protein daily.

Burke, Beal *et al.* (92) reported a long and careful study of the effect of maternal nutrition on the complications of pregnancy, fetal development, and infant health. Of the women receiving good or excellent diets 68 per cent had a normal prenatal course while only 42 per cent of those on poor or very poor diets had a normal prenatal course, and almost 50 per cent of the latter group had toxemia of pregnancy. The most striking differences were found in the infants. Of the children from the mothers on good or excellent diets 42 per cent were rated superior, while of those from the mothers on poor or very poor diets only 2.5 per cent were superior. The children of the mothers on the good diets had an average birth weight of eight pounds, eight ounces and an average length of 20.4 inches. The children from the mothers on the poor and very poor diets had an average birth weight of 5 pounds, 13 ounces and an average length of 18.6 inches. These studies serve to emphasize the magnitude and importance of the problem. Further ground work on the effect of specific nutrients on the offspring of experimental animals is needed so that more detailed clinical work can be done. Vitamin A studies by both Byrn & Eastman (93) and Lund & Kimble (94) show that giving vitamin A to the mother causes a rise in the maternal plasma vitamin level but does not affect that of the child. Lund & Kimble also found that low

maternal levels were not reflected in the fetus and the plasma vitamin A level in normal newborn infants was approximately one-half that of the normal adult values. Fetal plasma carotene varied regularly with the maternal plasma level. They feel that the newborn infant should be assured an adequate supply of vitamin A. Lockhart, Kirkwood & Harris (95) measured the thiamin excretion peaks in sixteen pregnant women and found that it was necessary to administer three times as much thiamin to secure excretion peaks in late pregnancy and early puerperium. They therefore suggest that thiamin requirements during pregnancy and lactation are three times those of non-pregnancy.

Military Rations

The composition and nutritive value of the most important of the Army rations was discussed by Berryman & Chambers (96). Rations now in general use are various types of field rations. The peace time "Garrison Ration" made up of thirty-nine components in prescribed quantities has limited use at the present time. Most troops within the continental limits of the United States receive "Field Ration A." This ration is roughly equivalent to a liberal civilian diet. Cost is the only limitation on the kind of food used and maximum use is made of fresh items. "Field Ration B" is sent with expeditionary forces as they leave this country and is intended for use where cooking and baking are performed with difficulty. It is similar to "Field Ration A" except that all perishable food is replaced by non-perishable, processed, or canned products. Menus are arranged in a ten-day cycle. About one-third of the vitamin C is obtained from lemon powder fortified with ascorbic acid. "Field Ration C" requires no refrigeration and is designed for use where no cooking or baking facilities are available and serious problems of supply exist. Foods are restricted as to type and number and the attainment of nutritional adequacy and palatability is difficult. The ration is not intended to be used over a long period of time as the sole source of nutriment but rather as a means of sustenance until contact is established with better facilities. This ration is provided in six cans per man per day. Three cans (M units) contain meat and vegetable components and the other three (B units) contain biscuits, soluble coffee, sugar, and hard candy. One of each of these units constitutes a meal and can be served either hot or cold. "Field Ration D" is a reserve ration of three 4-ounce chocolate bars intended to be

carried in the field pack or the pocket. Subsistence on this ration is obviously limited. The ration contains about 25 per cent fat and melts at about 120°F. It consists of chocolate, sucrose, dry skim milk, cacao fat, oat flour, vanillin, and thiamin hydrochloride (0.45 mg. per bar), and the three bars provide 1,800 calories. "Field Ration K" is a compact, light weight ration designed as a combat ration for use under serious difficulties in transport and supply where the soldier is far removed from a mess kitchen and where the ration may have to be used for a longer period of time than intended for "Field Ration C." "Field Ration K" consists of three units, one for each meal, packaged in small boxes impervious to water, chemical warfare agents, and vermin. The entire ration weighs only two pounds and does not require refrigeration. The ration (for three meals) is composed of canned ham and egg, canned meat, canned cheese preparations, two types of biscuit, a fruit bar, a type D chocolate bar, soluble coffee, bouillon powder, lemon powder, malted milk-dextrose tablets, sugar, chewing gum, and cigarettes. The ration provides fairly satisfactory nutrition with palatability and attractiveness to the soldier. The great military importance of these various rations is indicated by the differences in weight involved in transporting, preparing, and serving them. The total weight involved for 1,000 men for five days for field ration A is 56,580 pounds, for field ration B, 43,960 pounds, for field ration C, 27,400 pounds, and for field ration K, 13,350 pounds. The calculated nutritive value of the various rations as compared with the National Research Council's Recommended Allowances is given in Table III.

TABLE III

	N.R.C.*	Field Ration A	Field Ration C	Field Ration K
Calories	4,050	4,300	2,600–3,000	3,000–3,400
Protein (gm.)	70	130	120–140	115
Fat (gm.)	..	195	...	...
Carbohydrate (gm.)	..	510	...	...
Calcium (mg.)	800	1,000	500	1,000
Phosphorus (mg.)	..	2,000	...	2,000
Iron (mg.)	12	25	27	22
Vitamin A, I.U.	5,000	13,000	2,400	...
Thiamin (mg.)	2.15	3	1.5	3.0
Riboflavin (mg.)	3.12	2.8	1.6	2.5
Nicotinic acid (mg.)	21.5	30	26	25
Ascorbic acid (mg.)	75	130	27–33	55

* Based on ⅔ extreme activity and ⅓ moderate activity.

It is to be noted that the above values are for the ration as prescribed and do not take into account losses occurring in preparation and serving.

Enriched White Flour and Bread

Enriched white flour and bread were introduced in this country in 1941 in an effort to improve the nutritive value of American diets rapidly and effectively. Under a federal food distribution order (97) effective July 1, 1943, the enrichment of all white bread was required. Under the provisions of the Federal Food, Drug, and Cosmetic Act, amendments to the definition of enriched flour were made effective October 1, 1943 (98). These amendments provide that enriched flour shall contain in each pound not less than 2.0 mg. or more than 2.5 mg. of thiamin, not less than 1.2 mg. and not more than 1.5 mg. of riboflavin, not less than 16.0 mg. and not more than 20.0 mg. of niacin or niacin amide, and not less than 13.0 mg. and not more than 16.5 mg. of iron (Fe).

In a further amendment to the Food Distribution order (99) it is stated that effective January 16, 1944 "No baker shall make or sell any yeast-raised bakery product (except biscuits and crackers), or any doughnuts, crullers, or fried cakes, unless enriched to the extent that white flour is used as an ingredient." The effective date of this order has been postponed until May 1, 1944 or later.

Although there is at present no Federal order requiring the enrichment of flour the product has been made available by the millers voluntarily and can now be obtained throughout the United States. However, because this is a better product and slightly more expensive than lower-grade products the compulsory enrichment of family flour is needed in order for the nutritional benefits of the better product to reach those needing it most. A few state legislatures have enacted state laws requiring enrichment of flour.

An interesting statement of Federal policy on adding nutritive ingredients to foods was made by the Food and Drug Administration (100). It is stated that if processing staple foods removes significant quantities of nutritive factors present in the natural product and if the refined food is a suitable and efficient carrier of the factors, it is consistent with the promotion of honesty and fair dealing that refined foods be enriched on a restoration basis to the extent that restoration serves to correct deficiencies of such factors. However, when it is shown that restoration levels are too low to correct deficiencies, or

that deficiencies exist in other factors, for which the refined food is an efficient carrier, the inclusion of corrective quantities of nutritive factors in the enriched food may be required although such factors are wholly lacking in the natural product. Similar considerations may require the enrichment of unrefined foods.

The widespread use of enriched flour and bread in this country while other countries have introduced lightly milled products has renewed the interest in the old whole wheat versus white flour argument. It is obvious that from the point of view of human nutrition the enriched flour has a very satisfactory vitamin content. The presence of unidentified vitamins in whole wheat flour and the difference in protein value should be of little practical importance in comparison with the greater acceptability and wider range of usefulness of the white enriched flour.

The decalcifying effect of phytate has led Great Britain to the addition of calcium to their compulsory made, lightly milled flour. McCance, Edgecombe & Widdowson (101) have also shown that phytate interferes with iron absorption. Other possible disadvantages in the use of whole wheat flour are indicated by the studies of Rostorfer, Kochakian & Murlin (102) who found that a white bread containing 3.5 per cent milk solids, based on the flour, and made with a high-vitamin yeast had a digestion rate for protein 36 per cent faster and for carbohydrates 3 per cent faster than a peeled-wheat bread made from a flour containing about 98 per cent of the wheat kernel. A white bread made of "straight grade" white flour and containing 2.5 per cent milk solids, based on the flour, showed a digestion rate for protein 61 per cent faster and for carbohydrate 11 per cent faster than the peeled-wheat bread. It is also interesting to note that when the peeled-wheat bread was baked with a high-vitamin yeast the digestion rate of the white bread was only 39 per cent faster for protein and not at all faster for carbohydrate. These results indicate that there may be a real physiological basis for the failure of the general public willingly to replace white bread with whole wheat bread.

Sherwood (103) has given some interesting data bearing on the misconceptions concerning the proportion of thiamin in wheat that is contained in the wheat germ. The vitamins and minerals are unevenly distributed in the wheat kernel, with thiamin being most unevenly distributed. Of the total thiamin, the endosperm contains about 25 per cent, the germ about 15 per cent, and the outer layers of the kernel about 60 per cent. However, these values do not give a true picture of

the thiamin content of the various products of milling since a clean separation does not occur. Thus as the products of milling are prepared in commercial practice the germ constitutes only 0.2 per cent of the wheat and contains only 0.9 per cent of the total thiamin while "shorts" constitute 12.3 per cent of the wheat and contain 39.6 per cent of the thiamin. "Red Dog" constitutes 4.0 per cent of the wheat and contains 22.0 per cent of the thiamin. "Second clear" flour constitutes 4.5 per cent of the wheat and contains 10.0 per cent of the thiamin. "First clear" flour constitutes 7.0 per cent of the wheat and contains 3.9 per cent of the thiamin and "patent flour" constitutes 63.0 per cent of the wheat and contains 8.0 per cent of the thiamin. Bran constitutes 9 per cent of the wheat and contains 15.6 per cent of the thiamin. Thus in actual milling practice "Red Dog" and "shorts" together constitute only 16.3 per cent of the wheat but contain 61.6 per cent of the thiamin.

LITERATURE CITED

1. United Nations Conference on Food and Agriculture, Final Act with Section Reports, *Dept. of State Pub. 1948, Conf. Series 52* (U.S. Gov. Printing Office, 1943)
2. Various Authors, "Recommended Dietary Allowances," *Natl. Research Council Reprint Circ. Series,* **115** (Jan., 1943)

2a. Lanford, C. S., and Sherman, H. C., *Ann. Rev. Biochem.,* **12,** 398 (1943)

3. Boudreau, F. G., *Nutrition Revs.,* **1,** 321–26 (1943)
4. Parran, T., *U.S. Pub. Health Repts.,* **58,** 893–99 (1943)
5. Sebrell, W. H., *J. Am. Med. Assoc.,* **123,** 280–87 (1943)
6. Sebrell, W. H., *J. Am. Med. Assoc.,* **123,** 342–51 (1943)
7. Youmans, J. B., Patton, E. W., and Kern, R., *Am. J. Pub. Health,* **33,** 58–72 (1943)
8. Youmans, J. B., Patton, E. W., Sutton, W. R., Kern, R., and Steinkamp, R., *Am. J. Pub. Health,* **33,** 955–64 (1943)
9. Phipard, E. F., *Ann. Am. Acad. Pol. Soc. Sci.,* **225,** 66–71 (1943)
10. Stiebeling, H. K., *J. Am. Med. Assoc.,* **121,** 831–38 (1943)
11. Manning, I. H., Jr., and Milam, D. F., *Southern Med. J.,* **36,** 373–80 (1943)
12. Riggs, E., Perry, H., Patterson, J. M., Leeson, J., Mosley, W., and McHenry, E. W., *Can. Pub. Health J.,* **34,** 193–204 (1943)
13. Winters, J. C., and Leslie, R. E., *J. Nutrition,* **26,** 443–57 (1943)
14. Hardy, M. C., Spohn, A., Austin, G., McGiffert, S., Mohr, E., and Peterson, A. B., *J. Am. Dietet. Assoc.,* **19,** 173–81 (1943)
15. Kelly, H. T., and Sheppard, M., *New Engl. J. Med.,* **228,** 118–24 (1943)
16. Harris, R. S., Weeks, E., and Kinde, M., *J. Am. Dietet. Assoc.,* **19,** 182–89 (1943)

17. Sebrell, W. H., and Wilkins, W., *U.S. Pub. Health Repts.*, **58**, 803–13 (1943)
18. Kendrick, J. F., *U.S. Pub. Health Repts.*, **58**, 797–803 (1943)
19. Amyot, G. F., *U.S. Pub. Health Repts.*, **58**, 793–96 (1943)
20. Keys, A., *Federation Proc.*, **2**, 164–87 (1943)
21. Harper, A. A., Mackay, I. F. S., Raper, H. S., and Camm, G. L., *Brit. Med. J.*, **I**, 243–45 (1943)
22. Jenkins, G. N., and Yudkin, J., *Brit. Med. J.*, **II**, 265–66 (1943)
23. Keys, A., Henschel, A. F., Mickelsen, O., and Brozek, J. M., *J. Nutrition*, **26**, 399–415 (1943)
24. Williams, R. D., Mason, H. L., Power, M. H., and Wilder, R. M., *Arch. Internal Med.*, **71**, 38–53 (1943)
25. Barborka, C. J., Foltz, E. E., and Ivy, A. C., *J. Am. Med. Assoc.*, **122**, 717–20 (1943)
26. Najjar, V. A., and Holt, L. E., Jr., *J. Am. Med. Assoc.*, **123**, 683–84 (1943)
27. Wiehl, D., *Milbank Mem. Fund Quart.*, **20**, 329–66 (1942)
28. Borsook, H., Alpert, E., and Keighley, G. L., *Milbank Mem. Fund Quart.*, **21**, 115–57 (1943)
29. Goodhart, R., *J. Am. Med. Assoc.*, **121**, 93–97 (1943)
30. McIntire, J. M., Schweigert, B. S., Henderson, L. M., and Elvehjem, C. A., *J. Nutrition*, **25**, 143–52 (1943)
31. Schweigert, B. S., McIntire, J. M., and Elvehjem, C. A., *J. Nutrition*, **26**, 73–80 (1943)
32. Russell, W. C., Taylor, M. W., and Beuk, J. F., *J. Nutrition*, **25**, 275–84 (1943)
33. Oser, B. L., Melnick, D., and Oser, M., *Food Research*, **8**, 115–22 (1943)
34. Cheldelin, V. H., Woods, A. M., and Williams, R. J., *J. Nutrition*, **26**, 477–85 (1943)
35. Heller, C. A., McCay, C. M., and Lyon, C. B., *J. Nutrition*, **26**, 377–83 (1943)
36. Heller, C. A., McCay, C. M., and Lyon, C. B., *J. Nutrition*, **26**, 385–90 (1943)
37. Bing, F. C., *J. Am. Med. Assoc.*, **121**, 813–16 (1943)
38. Cowgill, G. R., *J. Am. Med. Assoc.*, **121**, 817–20 (1943)
39. Goodhart, R. S., *J. Am. Med. Assoc.*, **121**, 823–25 (1943)
40. Tennent, D. M., and Silber, R. H., *J. Biol. Chem.*, **148**, 359–64 (1943)
41. Cornbleet, T., Kirch, E. R., and Bergeim, O., *J. Am. Med. Assoc.*, **122**, 426–29 (1943)
41a. Mickelsen, O., and Keys, A., *J. Biol. Chem.*, **149**, 479–90 (1943)
42. Gant, O. K., Ransone, B., McCoy, E., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 276–79 (1943)
43. Lewis, K. H., Ham, W. E., and Jensen, W. I., *Proc. Soc. Exptl. Biol. Med.*, **52**, 33–35 (1943)
44. Newmann, F. W., Krider, M. M., and Day, H. G., *Proc. Soc. Exptl. Biol. Med.*, **52**, 257–60 (1943)
45. Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, **96**, 321–22 (1942)

46. West, H. D., Jefferson, N. C., and Rivera, R. E., *J. Nutrition*, **25**, 471–77 (1943)
47. Daft, F. S., Endicott, K. M., Ashburn, L. L., and Sebrell, W. H., *Proc. Soc. Exptl. Biol. Med.*, **53**, 130–31 (1943)
48. Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *U.S. Pub. Health Repts.*, **57**, 1559–66 (1942)
49. Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. J., *J. Biol. Chem.*, **148**, 721–22 (1943)
50. Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, **98**, 20–22 (1943)
51. Daft, F. S., and Sebrell, W. H., *U.S. Pub. Health Repts.*, **58**, 1542–45 (1943)
52. Totter, J. R., and Day, P. L., *J. Biol. Chem.*, **147**, 257–58 (1943)
53. Totter, J. R., Shukers, C. F., Kolsen, J., Mims, V., and Day, P. L., *Federation Proc.*, **2**, 72–73 (1943)
54. Wright, L. D., and Welch, A. D., *Science*, **98**, 179–82 (1943)
55. O'Dell, B. L., and Hogan, A. G., *J. Biol. Chem.*, **149**, 323–37 (1943)
56. Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, **97**, 404–5 (1943)
57. Stokstad, E. L. R., *J. Biol. Chem.*, **149**, 573–74 (1943)
58. Keresztesy, J. C., Rickes, E. L., and Stokes, J. L., *Science*, **97**, 465 (1943)
59. Wright, L. D., and Welch, A. D., *Science*, **97**, 426–27 (1943)
60. Welch, A. D., and Wright, L. D., *J. Nutrition*, **25**, 555–70 (1943)
61. Saslaw, S., Wilson, H. E., Doan, C. A., and Schwab, J. L., *Science*, **97**, 514–15 (1943)
62. Waisman, H. A., Rasmussen, A. F., Elvehjem, C. A., and Clark, P. F., *J. Nutrition*, **26**, 205–18 (1943)
63. Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, **26**, 361–75 (1943)
64. Ashburn, L. L., Daft, F. S., Endicott, K. M., and Sebrell, W. H., *U.S. Pub. Health Repts.*, **57**, 1883–91 (1942)
65. Holmes, H. N., *Southern Med. Surg.*, **105**, 393–94 (1943)
66. Berkman, S., *J. Infectious Diseases*, **71**, 201–11 (1942)
67. McCullough, N. B., and Dick, L. A., *J. Infectious Diseases*, **71**, 193–97 (1942)
68. Johnson, E. A., and Rettger, L. F., *J. Bact.*, **45**, 127–35 (1943)
69. Underkofler, L. A., Bantz, A. C., and Peterson, W. H., *J. Bact.*, **45**, 183–90 (1943)
70. Bovarnick, M. R., *J. Biol. Chem.*, **148**, 151–61 (1943)
71. Bovarnick, M. R., *J. Biol. Chem.*, **149**, 301–2 (1943)
72. Bovarnick, M. R., *J. Biol. Chem.*, **151**, 467–75 (1943)
73. Landy, M., Larkum, N. W., and Oswald, E. J., *Proc. Soc. Exptl. Biol. Med.*, **52**, 338–41 (1943)
74. Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, **97**, 265–67 (1943)
75. Tepley, L. J., Axelrod, A. E., and Elvehjem, C. A., *J. Pharmacol.*, **77**, 207–14 (1943)
76. Dorfman, A., and Koser, S. A., *J. Infectious Diseases*, **71**, 241–52 (1942)

77. Doyle, W. L., *Biol. Revs. Cambridge Phil. Soc.*, **18**, 119–36 (1943)
78. Hall, R. P., *Vitamins and Hormones*, **1**, 249–68 (Academic Press, Inc., New York, 1943)
79. Peterson, R. E., *J. Biol. Chem.*, **146**, 537–45 (1943)
80. Garnjobst, L., Tatum, E. L., and Taylor, C. V., *J. Cellular Comp. Physiol.*, **21**, 199–212 (1943)
81. Trager, W., *Science*, **97**, 206–7 (1943)
82. Trager, W., *J. Exptl. Med.*, **77**, 411–20 (1943)
83. Foster, C., Jones, J. H., Dorfman, F., and Kobler, R. S., *J. Nutrition*, **25**, 161–71 (1943)
84. Taylor, A., Pennington, D., and Thacker, J., *J. Nutrition*, **25**, 389–93 (1943)
85. Boelter, M. D. D., and Greenberg, D. M., *J. Nutrition*, **26**, 105–21 (1943)
86. Warkany, J., Nelson, R. C., and Schraffenberger, E., *Am. J. Diseases Children*, **64**, 860–66 (1943)
87. Warkany, J., Nelson, R. C., and Schraffenberger, E., *J. Bone Joint Surg.*, **25**, 261–70 (1943)
88. Warkany, J., and Schraffenberger, E., *Proc. Soc. Exptl. Biol. Med.*, **54**, 92–94 (1943)
89. Warkany, J., Reported at meeting Region 2, *Am. Acad. Pediat.* (Nov., 1943)
90. Ebbs, H. J., *J. Am. Med., Assoc.*, **121**, 339–45 (1943)
91. Bethell, F. H., Blecha, E., and Van Sant, J. H., *J. Am. Dietet. Assoc.*, **19**, 165–72 (1943)
92. Burke, B. S., Beal, V., Kirkwood, S. B., and Stuart, H. C., *Am. J. Obstet. Gynecol.*, **46**, 38–52 (1943)
93. Byrn, J. N., and Eastman, N. J., *Bull. Johns Hopkins Hosp.*, **73**, 132–37 (1943)
94. Lund, C. J., and Kimble, M. S., *Am. J. Obstet. Gynecol.*, **46**, 207–21 (1943)
95. Lockhart, H. S., Kirkwood, S., and Harris, R. S., *Am. J. Obstet. Gynecol.*, **46**, 358–65 (1943)
96. Berryman, G. H., and Chambers, W. H., *Gastroenterology*, **1**, 335–46 (1943)
97. *Federal Register*, **8**, 8387–88 (1943)
98. *Federal Register*, **8**, 9115–16 (1943)
99. *Federal Register*, **8**, 16777–78 (1943)
100. *Federal Register*, **8**, 9170 (1943)
101. McCance, R. A., Edgecombe, C. N., and Widdowson, E. M., *Lancet*, **245**, 126–28 (1943)
102. Rostorfer, H. H., Kochakian, C. D., and Murlin, J. R., *J. Nutrition*, **26**, 123–38 (1943)
103. Sherwood, R. C., *Am. J. Pub. Health*, **33**, 526–32 (1943)

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THE NUTRITIONAL DEFICIENCIES IN FARM MAMMALS ON NATURAL FEEDS

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The deficiencies of farm mammals which are most widespread and of greatest economic importance are not usually manifest by clinical symptoms but are evidenced by a decrease in the production of work, wool, or human food. Except under extreme conditions, such deficiencies as energy and protein are seldom detected by clinical symptoms. The deficiencies which are most evident are frequently exaggerated beyond their rightful economic importance. Many nutritional deficiencies in livestock have been produced under laboratory conditions with unnatural rations but these disturbances may or may not apply to natural conditions on the farm or range. The extent of the problem is also indicated from the evidence which is accumulating that many nutritional disturbances are the result of depletion or primary deficiencies in the soil, or to the climatic conditions under which the feedstuffs are produced, and the seasonal variations in the occurrence of some deficiency diseases. The value of the feedstuff depends further on the species of plant, on the species of animal, on the purpose for which the animal is fed, and on the economics of the relative cost of feed in comparison to the return on the product. It is therefore impossible to give a complete and critical account of the nutritional deficiencies recognized in farm animals on natural feeds because of the fragmentary data on the chemical composition and nutritive value of the rations as fed under farm or range conditions.

Energy

The lack of sufficient energy in the ration of farm animals on natural feeds is the most important deficiency from the standpoint of economical production of milk, meat, wool, and work. On the marginal farms and in many parts of the range country, feed energy is one of the limiting factors for successful reproduction (1). The deficiency of energy may be due to crop failure, adverse climatic conditions, insect pests, or to a failure to adjust the livestock population to the productivity of the land. The amount of energy it is necessary to feed above maintenance depends on the efficiency of the animal as a con-

verter, on the relative cost of the feed, and on the economic return. The most expensive part of the ration of livestock is calories. Under range conditions, beef cattle and sheep are seldom able to eat sufficient forage for finishing. Slow growth on range grass alone may produce greater profits than when expensive supplements are fed to meet the animal's physiological needs for optimum growth and fattening. When pastures are very short the animals may not be able to gather the requisite quantity of food in the time available (2). Even if a reserve stand of long pasture is available it will be dry and fibrous, of relatively low nutritive value, and will not provide sufficient nourishment for milk production even when the cows are fed to capacity.

The availability of digestible calories is variable, depending upon the stage of maturity of the plant at the time of harvest and on the species of animal to which it is fed. Pasture plants harvested prior to the bud stage show high coefficients of digestibility by all classes of farm animals but after the bud stage is reached the digestibility of the dry matter decreases (3, 4). The depression in digestibility may be associated with an increase in methoxyl groups in the lignin (5). In many areas, pastures at the most highly digestible stage fail to meet the caloric need of dairy cattle for maintenance and the most economic milk production. This may also apply to finishing beef cattle and sheep on pasture. Pasture cattle dress materially lower than cattle fattened on grain and pasture and the meat has inferior qualities (6). Steers will make cheaper gains on pasture without grain but they will lack finish (7). Although the pastures are good the steers cannot consume sufficient forage to meet their needs for finishing. Home-grown grains will usually supplement pastures for both dairy cattle, beef steers, and fattening sheep. Unless grain is fed as a supplement, the most marked energy deficiency occurs when hot dry weather reduces the growth of pasture forage.

During the stall-feeding period many farm animals consume large quantities of roughage that is deficient in digestible calories. This is not the case with swine because these animals are usually fed cereal grains liberally, with the exception of brood sows.

The stage of maturity of the roughage at the time of harvest and the losses due to curing appear to determine its nutritive value. When dairy cattle are changed from alfalfa alone to good pasture, there is an increase in milk production comparable to the addition of from two to twelve pounds of grain per day (8). The substitution of young artificially dried grass for hay or grain in the ration on the basis of

an equal amount of total digestible nutrients produces an increase in milk and fat production (9 to 13). The deficiencies of an all-roughage ration for milk production have been indicated by Graves *et al.* (14). The value of grain as a supplement to roughage rations from the standpoint of milk production has been pointed out (15). The added grain supplies both additional calories and milk-stimulating factors.

When an isocaloric amount of the total digestible nutrients in alfalfa hay is replaced by corn, after milking cows have been depleted of their lactation factors on alfalfa hay alone, there is a marked increase in milk production against the natural decline in lactation (16). These results indicate that the digestible nutrients of alfalfa hay are deficient in some unknown factor or factors needed for lactation. The addition of either corn starch or glucose fails to stimulate milk production but when corn replaces an equicaloric amount of these supplements, there is a marked increase in milk production (17). The superior value of young pasture herbage and early cut hay compared with late cut hay for milk production is probably due to the presence of an unknown lactation-stimulating factor or factors. Since swine are usually fed concentrates as a basis of their ration, the lactating brood sow probably does not suffer from a deficiency of the unknown factor or factors.

Protein

Protein deficiency is usually manifested by a reduction in growth, fattening, or in milk production. Since economical livestock production is based on the use of roughages, information concerning their amino acid content is essential (18). Cystine deficiency in sheep was reviewed in the light of the role of methionine as the essential amino acid necessary for wool production (19). Increasing the amount of protein results in a favorable influence on the quality (fineness) and quantity of wool grown by high-producing sheep. Since ruminants and horses are large consumers of roughage the nonprotein nitrogen assumes some significance due to the possibility of protein synthesis by microfauna and microflora in the rumen and caecum (20). The young farm animals which suckle their dams for various lengths of time do not suffer from a protein deficiency during the early months of life. The exception to this is the dairy calf which is taken away from its dam at about two days of age (21, 22).

In areas of limited rainfall, which include a large percentage of the total grazing land throughout the world, the herbage is usually low in protein. The native pastures in South Africa frequently do not con-

tain enough protein to meet the requirements of grazing livestock (23, 24). Bluestem grass grown in Oklahoma contains less than 4 per cent protein (25). The forage grasses in sections of Texas frequently do not supply enough protein for the maintenance requirements of grazing cattle (26). The protein content of range plants in California, Arizona, and Utah showed wide variations (27, 28, 29). The protein content is so low during a large part of the year that it is necessary to feed legume hay. The liberal feeding of leguminous hay or silage and home-grown grains during the winter usually furnishes ample protein for growing and fattening steers and breeding cows. When legumes are not available a protein concentrate should be fed for economical production of all classes of beef cattle.

Low protein and inadequate phosphorus in the feeding regimen of breeding ewes must be avoided if the condition of the ewe is to be maintained and a satisfactory lamb crop obtained (30). On many farms sheep and cattle are fed low protein roughages during the stall-feeding period without a protein supplement. This type of ration is too low in protein except for maintenance. The possibility of a protein deficiency occurring under range conditions when the protein content of the grasses is very low is apparent. Sheep suffer from a protein deficiency on natural pastures in South Africa from April to September unless protein supplements are supplied (23, 31). The protein content of the grasses varies from 3.3 to 4.0 per cent. Very little information is available on the requirements and deficiencies of horses on natural forages. Swine are the farm mammals most likely to suffer from a deficiency of protein both from the standpoint of quality and quantity except when skim milk is available or in regions where peanuts are grown. Brood sows can be maintained on alfalfa and cereal grains but when sows are suckling pigs a protein supplement should be fed (32). One half of the protein for weanling pigs should be supplied from animal sources (33). The cereal grains do not contain a sufficient quantity of protein and the quality is believed to be insufficient. The high-quality protein in the past has been supplied from animal sources; however, for optimum pork production, the amount of purchased protein concentrates can be reduced by a greater use of pastures which not only furnish 15 to 20 per cent protein on the dry basis but also contain protein of high biological value. In sections where peanuts are fed, sufficient protein is furnished but a small amount of animal protein is recommended (34). Soybeans have a very limited use in swine feeding because they produce an inferior

quality of pork (35). The amount of animal protein can be reduced when toasted soybean oil meal is used as the principal source of protein (36, 37).

Minerals

Salt.—The salt content of feeds from plant origin is usually low in both sodium and chlorine. The addition of salt to swine rations which do not contain animal protein results in an increase in the rate and economy of gain in weight (38, 39, 40). Pigs fed exclusively on peanuts as the principal source of protein are unthrifty and make uneconomical gains unless salt is added to the ration (41). Salt deficiency in cattle on natural feeds is common after the onset of lactation (42). A deficiency of salt in the ration of the calf is reflected in an unthrifty condition and a harsh coat. Pregnant ewes appear to require salt (43). The salt requirement of the working horse in warm weather is high (44). Camels develop skin necrosis and lameness on salt deficient rations (45).

Iodine.—There are certain areas around the Great Lakes and other sections of the United States in which the soil, plants, and water are sufficiently low in iodine to produce a high incidence of goiter in farm animals. There are also marginal areas in which the deficiency is not extreme enough to produce frequent trouble but does lead to some difficulty (46, 47, 48). Soils in the goitrogenic regions in New Zealand show little correlation between the iodine content of the soil and the herbage growing on them, but cow's milk from goiter-free areas contains three times as much iodine as does that from affected areas (49, 50). The forages of livestock in the interior of Norway do not supply enough iodine for normal health (51). The minimum requirements of farm animals for iodine has not been reported but the indiscriminate use of an iodine supplement is not recommended (47, 52).

Phosphorus.—Phosphorus deficiency is probably the most extensive mineral deficiency observed in cattle and sheep on natural rations. The possibility of a phosphorus deficiency depends upon the phosphorus content of the roughage and grain. The use of root crops tends to increase the possibility of a phosphorus deficiency. When roughages contain 0.18 per cent or more of phosphorus on the dry basis, there is little likelihood of a phosphorus deficiency in dairy cows even when corn is fed as the only concentrate (53). The extent of phosphorus deficiency in dairy cows is probably small in most parts of the world.

Many soils and pastures in Queensland are so deficient in phos-

phorus that bone meal has to be fed in order to increase fertility, secure earlier maturity, and prolong the life of older cows (54). The higher savannahs in British Guiana are valueless for cattle grazing because of the extremely low phosphorus and calcium contents of the grasses (55). The Uruguayan grassland regions in which osteomalacia in cattle is common are deficient in phosphorus and calcium. The distribution of phosphatic licks has not only controlled the disturbance but has also increased the calf crop by approximately 20 per cent per year (56, 57, 58). Native Jamaican Zebu cattle raised on phosphorus-deficient forage do not show the deficiency diseases ordinarily manifested by the imported breeds (59). The poor quality of Malabar cattle in southern India is due to mineral deficiencies in the soil and herbage (60). Osteomalacia was found to be prevalent in cattle of all ages in the province of Corrientes, Argentina, where soils and forage are low in calcium and phosphorus (61). Du Toit *et al.* (62, 63) report that all of the South African pastures composed mainly or wholly of grasses are deficient in phosphorus and protein from five to nine months of the year. These pastures do not contain enough phosphorus for gestation or lactation at any time of the year. The natural pastures in Nigeria are so deficient in calcium and phosphorus that they do not maintain the nomadic cattle during the six-month dry season (64). Under open range conditions in Bechuanaland nursing cows require a phosphorus supplement in order to supply enough milk for their calves to be normal at weaning age (65). A disturbance in ruminants in certain areas in Norway known as *"slikkesyken"* is associated with a low phosphorus content of hay and fodder (66).

Calves maintained over periods of two to four years on a prairie hay ration, deficient in phosphorus and protein, grew at a subnormal rate, developed coarse coats, disproportionately large heads, and had an emaciated appearance. Chronic phosphorus deficiency was indicated by hypophosphatemia (67). The forage grasses in the East Timber country, Texas, supply enough calcium to grazing animals but usually not enough phosphorus (26). The range grasses in Sutton and Edwards counties are deficient in phosphorus for cattle from September to February but sheep and goats receive a fair supply for nine months of the year (68). Cattle on phosphorus deficient ranges in southern Texas respond to bone meal feeding by preventing hypophosphatemia, by markedly increasing the calf crop, and by increasing the weaning weights of calves about 70 per cent due to the increased milk production of the dams (69). The range forage around Las Cruces, New

Mexico, is adequate in calcium but is deficient in phosphorus during most of the year (70). The lack of phosphorus is particularly serious for gestating and lactating cows. Heifers that had access to bone meal were much more active sexually than heifers that did not have access to bone meal (71). Supplementary feeding of calcium and phosphorus to cattle and sheep on ranges where deficiency symptoms are not in evidence resulted in smaller death losses in newborn calves, a larger number of calves weaned, greater weight of calves and lambs at weaning time, greater body weight as yearlings, and higher wool production from range ewes (72). The feeding of mineral supplements to range cattle in certain sections of Arizona did not pay even though the grasses were deficient in phosphorus for five months of the year (73).

Phosphorus supplements are unnecessary for sheep when the herbage contains from 0.07 to 0.12 per cent phosphorus on the dry basis (74). Sheep under range conditions in Idaho did not show phosphorus deficiency symptoms but the level of blood phosphorus was low (75). Hoflund (76) found that "*skravel*" disease of sheep in Sweden was associated with the hay grown on phosphorus-deficient soils. The native sheep were less susceptible to the disorder than the larger, more highly bred type of imported sheep. Phosphatic fertilizers improved the quality of the hay to such an extent that the disease disappeared. When swine receive a reasonable amount of calcium and vitamin D there is no evidence of a phosphorus deficiency when they are fed natural rations.

Calcium.—The possibility of calcium deficiency occurring among farm animals depends on the calcium content of the forage, the amount of roughage consumed, the ratio of calcium to phosphorus, the amount of vitamin D in the feed, the environment, and on the product produced, such as milk, meat, or wool. The experiment frequently referred to in the literature, which indicates that lactating cows suffer from a lack of calcium, was not characteristic of common dairy cattle rations because very little roughage was fed (77). Calcium may be a secondary deficiency when cattle are suffering from anorexia due to a phosphorus or cobalt deficiency. The liberal feeding of roughage containing 0.3 per cent calcium on the dry basis furnishes ample calcium for reproduction and lactation (78, 79).

Sheep under pasture conditions do not ordinarily suffer from an uncomplicated calcium deficiency although ewes brought into certain districts in Western Australia could not be used for breeding stock after the first year because the second crop of lambs developed rachitic-

like symptoms (80). This condition may be associated with copper deficiency. The feeding of fattening lambs from two to five grams of calcium per day as a supplement to a sorghum ration resulted in an increase in appetite for roughage and the lambs made larger gains and better finish (81).

There is very little information in the literature on the calcium and phosphorus requirements of the horse, but outbreaks of abortion in mares on calcium deficient rations have been reported (82, 83). Army horses and mules in Panama suffering from lameness made marked improvement after about one-third of the low-calcium hay was replaced with alfalfa hay (84). A degree of calcium deficiency in the rations of race horses has been suggested (85). Calcium deficiency in the local grasses was the cause of equine osteodystrophic diseases in the Dutch East Indies (86). The calcium deficiency manifestations in the horse are due, for the most part, to feeding rations high in phosphorus.

When pigs are fed rations consisting largely of cereal grains, in dry lot, with no skim milk or tankage, a calcium deficiency is often observed. Winter conditions aggravate the calcium deficiency. Many cases of rickets have been observed on farms where inadequate rations were fed (87). Swine rations containing 0.41 per cent calcium and 0.3 per cent phosphorus give better results than when lower levels of phosphorus are fed (88). Growing, fattening, and lactating sows develop posterior paralysis when kept exclusively on peanut fields during the fall and winter months (41). The addition of calcium carbonate and salt prevent the deficiency symptoms and promote normal growth. The need for an additional calcium supplement is indicated except when brood sows are fed liberal amounts of sun-cured legume hay.

Cobalt.—The essentiality of cobalt in the ration of sheep and cattle has been shown (19, 89, 90). Pasture plants taken at monthly intervals show that the cobalt content tends to increase in the late autumn when plant growth is retarded. In areas where sheep are affected the cobalt content of the pastures in the spring and summer falls below 0.08 p.p.m. (91). Grasses grown on highly calcareous soils are generally low in cobalt (92). "Coast disease" in sheep and cattle in Tasmania is due to a dual deficiency of copper and cobalt. Cobalt alone will keep the sheep alive but it will not lead to full recovery unless copper is administered (93, 94). A seasonal variation of an uncomplicated cobalt deficiency in sheep is reported on one type of soil in the "coasty" region in South Australia formerly used to grow cereal crops (95).

Lambs make good growth during the early part of the year but soon showed signs of unthriftiness, then emaciation and death. Later in the season mature sheep show the same symptoms with high mortality rate.

Soils in Scotland where "pining" occurs usually contain less than 5 p.p.m. of cobalt. The value of cobalt fertilizers has been confirmed (96). "Vinguish" in sheep in Ayrshire is an uncomplicated cobalt deficiency which can be prevented and cured by cobalt therapy (97). Sheep in sections of western Alberta become unthrifty when fed non-leguminous hays and ground oats. The administration of cobalt to the affected sheep results in a rapid increase in weight and a marked improvement in condition. Bowstead *et al.* (98, 99) confirmed their earlier findings and reported an impairment in the reproductive capacity of the sheep. The ewes became so unthrifty that they were unable to nourish their lambs. Their wool was also weak in fiber. The administration of 6 to 8 mg. of cobalt daily restored sheep to normal health.

The so-called "Grand Traverse" disease occurring in cattle in several areas in Michigan is due to the low cobalt content of locally grown hays and cereal grains. The disturbance is seasonal in character and occurs on both light sandy soils and on clay loam soils. When cattle are moved to nearby unaffected pastures the disease disappears or it is quickly amenable to cobalt therapy (100).

Copper.—Bennetts *et al.* (101) pointed out that the mortality of "falling disease" in cattle was associated with a very low copper content of the pastures. The affected pastures contain as little as 1 p.p.m. of copper on the dry basis as compared to 5 to 15 p.p.m. on healthful pastures. Further investigations showed that "falling disease" is the terminal manifestation of a severe copper deficiency (102). The incidence of copper deficiency was associated with "good clover years" and not with unusually dry or "poor clover years." The copper content of pastures decreases as the herbage approaches maturity (103). Hemoglobinuria in calves and sporadic cases of "red water" in mature cows were associated with the low copper content of the forage (104). The incidence varied from farm to farm and from year to year and the mortality varied from 5 to 80 per cent. The most obvious symptom of copper deficiency in sheep is a progressive change from normal wool to straight, glistening, lustrous wool, known to the wool trade as "straight steely" wool (105). The disorder is cured by supplying the sheep with copper and cobalt either as a drench or as salt licks.

Attempts to breed sheep producing "straight steely" wool result in the occurrence of ataxia in the offspring. "Stringy" wool appears to be the earliest and most prominent sign of copper deficiency even when the deficiency is not sufficiently severe to cause ataxia in lambs (106). The daily intake of 15 mg. of copper will eliminate the disease. In certain areas in England ataxia is the most serious lamb disease. Ewes give birth to healthy and affected lambs in consecutive years on the same pasture (107). The incidence of ataxia or "swayback" is low in lambs from ewes which had access to licks containing 0.3 per cent copper during the gestation period.

Certain similarities have been indicated between the disease in England and Australia and an ataxia described by workers in other countries (108, 109, 110). A deficiency disease in cattle in Norway known as *"slikkesyken"* is associated with the low copper content of hay and fodder grown along the coast (66). Young cattle and fresh cows are especially susceptible during the late winter and early spring months. The ingestion by cattle of less than 1.5 to 3.0 p.p.m. of copper in forage dry matter caused copper deficiency symptoms although the deficiency may occur with several times this amount of copper (111). Blood, liver, and hair are the only tissues that show consistent correlation with the copper intake. Cause of *"lecksucht"* has been confirmed and extended to indicate that the therapeutic value of copper alone is just as effective as a complex mineral mixture (112), that the composition of the soil does not offer an explanation as to the cause of the disease (113) but that top-dressing the pastures with copper sulfate is effective in preventing the disease in young cattle (114). A progressive unthriftiness, hemoglobinuria, and a condition of the blood (in which as many as 35 per cent of the red blood cells show a thorn-apple shape within two days) have been observed after the cattle were placed on peatland pasture (115, 116).

Iron and zinc.—Iron was reported to be deficient in the rations of cattle in the West Indies during the stall-feeding season. The symptoms were alleviated by supplementing the ration with iron oxide and copper sulfate (117). A deficiency of iron in the soil and vegetation in certain regions in Uruguay has necessitated the use of iron-containing licks to maintain the grazing cattle (58). A nutritional anemia in cattle observed in a section of Massachusetts responded to iron therapy (118). The forage was found later to be low in cobalt as well as in iron (119). The acid soils in northern Sweden are indicated as being responsible for an anemia occurring in horses (120). Suckling

pigs should be provided with a source of iron within a few days after birth to insure normal development and resistance to disease (121, 122). A decrease in the number of still-births can be obtained by feeding iron salts to sows during gestation (123).

Sterility of unknown origin in cows has been reported to respond to zinc therapy (124).

Vitamins

Vitamin A. — Vitamin A deficiency is not commonly observed among farm mammals except under range conditions when the herbage is dry and bleached (125), or during drought periods (126), or on farms during the stall-feeding period when straw and other low carotene roughages are used. Farm animals have the ability to store vitamin A (127), therefore, if they have had access to good pasture that was high in carotene they will start the stall-feeding period with a large reserve (78, 128). Roughages used during the winter are highly variable in carotene content because of the losses incurred during the curing process and during storage (78, 129).

Diarrhea was observed in newborn calves when the ration of the dam was low in carotene (130). Calves born of cows whose colostrum is low in vitamin A are more liable to suffer from infections than the calves from cows whose colostrum is high in vitamin A (131). Ordinarily colostrum is a good source of vitamin A (132 to 135). Steers fed old corn and bleached alfalfa hay for fourteen months manifested a vitamin A deficiency which was cured by changing to new corn and cod liver oil (136).

The mortality of newborn calves was high in a herd of cattle fed wheat straw, oats, and yellow corn but the losses were reduced when the dams were changed to clover hay, corn, oats, and minerals (137). Many cows under farm conditions in Germany gave birth to blind calves which was probably due to a vitamin A deficiency (138, 139). Blindness and other symptoms of vitamin A deficiency are widespread among cattle in India (140, 141). Keratitis in five- to ten-month-old beef calves that had been overwintered on dry sorghum and corn stalks was cured within two weeks after they had access to well cured alfalfa hay, yellow corn, and minerals (142).

Vitamin A deficiency occurs among rams on pasture during the drought season in New South Wales and Queensland. The administration of vitamin A or carotene prevented night blindness and semen degeneration (143). During drought years, lambs suffer from a nutri-

tional keratitis due to a vitamin A deficiency but they make rapid recovery when green hay is available. Nutritional keratitis may also be a limiting factor in lamb production (144). Urinary calculi in sheep is not due to a vitamin A deficiency (145).

Numerous instances have been reported which indicate that army horses suffered from a vitamin A deficiency when the ration consisted of straw or poor hay and grain, other than yellow corn (146 to 149). Access to green pasture or the feeding of Virtanen (A.I.V.) silage or cod liver oil prevented the disturbance. The causative factor in equine joint involvements and characteristic lesions in the articulating cartilage is not due to a lack of vitamin A (150).

Pigs raised indoors on a grain protein concentrate and a mineral mixture developed symptoms of vitamin A deficiency (151). Sows fed a mixed grain ration gave birth to pigs which had impaired vision and died within three to five days (152). These rations are probably deficient in vitamin D as well as vitamin A. Vitamin A deficiency in swine can be prevented by feeding good quality alfalfa hay (153).

Vitamin-B complex.—Most of the members of the vitamin-B complex are synthesized in the rumen and caecum by the flora and fauna of farm mammals, other than swine (20). There is the possibility of a deficiency of some member of the B complex in the young prior to the establishment of a rumen flora and also in older animals when the flora is abnormal. The desirability of balancing the food of rumen bacteria is indicated by the increased synthesis of riboflavin when corn was added to an all-roughage ration (154). Diarrhea in calves responds favorably to the administration of nicotinic acid and vitamins A and C (130, 155). A nutritional disturbance in calves under field conditions characterized by poikilocytosis was cured by feeding either yeast or pyridoxin (156). A vitamin B_1 deficiency in young cattle, manifest by a sagging back, ataxic gait, and irregular heart action, responded favorably to oral administration of thiamin (157). A nervous condition in pregnant ewes was alleviated by feeding yeast and good hay (157, 158). Although swine are known to require thiamin, riboflavin, pantothenic acid, pyridoxin, and choline, a deficiency of any of these vitamins is not known to occur on natural rations. Several diseases of pigs under field conditions, however, have responded to nicotinic acid therapy (159, 160, 161).

Ascorbic acid.—The earlier work on swine and cattle indicating that these animals can synthesize vitamin C has been confirmed (162). There are several references in the literature referring to the occur-

rence of scurvy in domestic animals but the first proof that these animals might not be able to synthesize adequate ascorbic acid for optimum physiologic functioning was reported by Phillips *et al.* (163). From a study, under field conditions, of the ascorbic acid content of the semen and blood plasma of normal and sterile bulls, and the blood plasma of cows, it was concluded that ascorbic acid is intimately involved in the production of virile sperm in the bull and that it is vitally concerned in the physiology of reproduction in the female bovine (164). Ingested ascorbic acid is destroyed in the rumen of the bovine (165). Bulls which have low blood ascorbic acid values and poor breeding performance are usually fed poor quality roughages and cows fed good roughages have a higher level of ascorbic acid in the blood than cows receiving poor roughages (166). The synergy between vitamin A and ascorbic acid on the reproductive efficiency of farm mammals needs clarification (167). Since many bulls and cows respond to ascorbic acid therapy on natural rations supplying ample carotene, it appears that other factors may also be involved in maintaining normal ascorbic acid synthesis. Recent work has shown that the feeding of chlorobutanol to the bovine results in a marked increase in the level of ascorbic acid in the blood plasma (168) and can restore fertility to sterile bulls (169). The ascorbic acid content of colostrum of cows and ewes is reported to be much higher than in the following milk (170) but recent work indicates that an ascorbic acid supplement may be needed by the young calf (155). The possibility of insufficient ascorbic acid synthesis in other farm animals is meager. Beneficial results were obtained by injecting ascorbic acid into unthrifty pigs (171). Stallions with low breeding efficiency have lower plasma ascorbic acid values than normal stallions and good breeding performance is associated with good pastures (172).

Vitamin D.—Farm mammals under natural feeding conditions received adequate vitamin D from solar radiation and sun-cured roughage (173, 174). The amount of vitamin D in the roughage depends upon the intensity of solar radiation during the curing process (175, 176, 177). Any disturbance which results in anorexia may reduce the intake of sun-cured hay and induce vitamin D deficiency symptoms (178). Rickets observed in beef calves is due to heavy feeding on concentrates, low hay intake, rapid growth, and indoor confinement (179). Horses and mules consume enough sun-cured hay to protect them from symptoms of vitamin D deficiency. Fitch (180) reviewed the literature concerning the effect of the ration on bone changes in sheep but only found a few cases that manifested vitamin D deficiency.

Rickets in pigs is reported to be common during the winter months (181). Pigs fed indoors and allowed voluntary access to an outside runway did not receive enough solar radiation to prevent rickets during December and January but an enforced forty-five-minute exposure daily to January sunshine was sufficient to cure rickets (177). The vitamin D requirements of swine vary with the level of calcium and phosphorus in the ration.

Vitamin E.—Several investigations have reported beneficial results from treating sterile cows with a vitamin E supplement (182 to 185). Vitamin E was found to be nonessential for normal reproduction in sheep and goats (186). The field observations which indicate a positive role of vitamin E in the treatment of sterility in farm animals are probably of questionable value because no control animals were studied. Many cases of sterility in farm animals are cured spontaneously.

LITERATURE CITED

1. PHILLIPS, P. H., "Third Report of the Committee on Animal Nutrition," *Natl. Research Council Circ.*, **112**, (1942)
2. RIDDET, W., CAMPBELL, I. L., MCDOWALL, F. H., AND COX, G. A., *New Zealand J. Sci. Tech.*, **23A**, 80–112 (1942)
3. SOTOLA, J., *J. Agr. Research*, **61**, 303–11 (1940)
4. BURKITT, W. H., *J. Agr. Research*, **61**, 471–79 (1940)
5. PHILLIPS, M., DAVIS, B. L., AND WEIHE, H. D., *J. Agr. Research*, **64**, 533–46 (1942)
6. BULL, S., SNAPP, R. R., AND RUSH, H. P., *Illinois Agr. Expt. Sta. Bull.*, **475**, 227–56 (1941)
7. BRANAMAN, G. A., FICK, H. A., AND HARRISON, C. M., *Mich. Agr. Expt. Sta. Quart. Bull.*, **25**, 100–5 (1942)
8. WILLARD, H. S., *Wyoming Agr. Expt. Sta. Bull.*, **237** (1940)
9. WATSON, S. J., "The Science and Practice of Conservation: Grass and Forage Crops," *Fertiliser, Feeding Stuffs Farm Supplies J.* (London, 1939)
10. NEWLANDER, J. A., *Vermont Agr. Expt. Sta. Bull.*, **350** (1933)
11. NEWLANDER, J. A., AND JONES, C. H., *Vermont. Agr. Expt. Sta. Bull*, **348** (1932)
12. CAMBURN, O. M., AND JONES, C. H., *Vermont Agr. Expt. Sta. Bull.*, **446** (1939)
13. CAMBURN, O. M., *Vermont Agr. Expt. Sta. Bull.*, **359** (1933)
14. GRAVES, R. R., BATEMAN, G. Q., SHEPHERD, J. B., AND CAINE, G. B., *U.S. Dept. Agr. Tech. Bull.*, **724** (1940)
15. JENSEN, E., KLEIN, J. W., RAUCHENSTEIN, E., WOODWARD, T. E., AND SMITH, R. H., *U.S. Dept. Agr. Tech. Bull.*, **815** (1942)
16. HUFFMAN, C. F., *J. Dairy Sci.*, **21**, 101 (1938)
17. HUFFMAN, C. F., AND DUNCAN, C. W., *Mich. Agr. Expt. Sta. Quart. Bull* (In press)
18. LUGG, J. W. H., *J. Council Sci. Ind. Research*, **14**, 209–14 (1941)
19. MARSTON, H. R., *Ann. Rev. Biochem.*, **8**, 557–78 (1939)
20. GOSS, H., *Nutrition Abstracts & Revs.*, **12**, 531–38 (1943)
21. SAVAGE, E. S., AND MCCAY, C. M., *J. Dairy Sci.*, **25**, 595–650 (1942)
22. SAVAGE, E. S., KRAUSS, W. E., AND MEAD, S. W., "Fifth Report of the Committee on Animal Nutrition," *Natl. Research Council*, unnumbered mimeo. (1942)
23. HENRICI, M., *Union S. Africa Dept. Agr. Sci. Bull.*, **115** (1932)
24. DU TOIT, P. J., LOUW, J. G., AND MALAN, A. I., *Onderstepoort J. Vet. Sci. Animal Ind.*, **5**, 215–70 (1935)
25. TAYLOR, B. R., *Oklahoma Agr. Expt. Sta. Mimeo. Circ.*, **98** (1943)
26. FRAPS, G. S., AND FUDGE, J. F., *Texas Agr. Expt. Sta. Bull.*, **582** (1940)
27. GORDON, A., AND SAMPSON, A. W., *Calif. Agr. Expt. Sta. Bull.*, **627** (1939)
28. STANLEY, E. B., AND HODGSON, C. W., *Ariz. Agr. Expt. Sta. Tech. Bull.*, **73** (1938)
29. ESPLIN, A. C., GREAVES, J. E., AND STODDART, L. A., *Utah Agr. Expt. Sta. Bull.*, **277** (1937)

30. Miller, R. F., Hart, G. H., and Cole, H. H., *Calif. Agr. Expt. Sta. Bull.*, **672** (1942)
31. Smuts, D. B., Marias, J. S. C., and Louw, J. G., *Farming in S. Africa*, **15**, 229–32 (1940)
32. Work, S. H., Henke, L. A., and Harris, L. E., *J. Animal Sci.*, **1**, 72 (1942)
33. Crampton, E. W., *Proc. Am. Soc. Animal Production*, **32**, 144–47 (1939)
34. Massey, Z. A., *Georgia Agr. Expt. Sta. Circ.*, **118** (1939)
35. Carroll, W. E., *Illinois Agr. Expt. Sta. Circ.*, **369** (1934)
36. Ellis, N. R., and Zeller, J. H., *J. Animal Sci.*, **2**, 374 (1943)
37. Robison, W. L., *J. Animal Sci.*, **2**, 376 (1943)
38. Evvard, J. M., Culbertson, C. C., Hammond, W. E., and Wallace, O. W., *Iowa Agr. Expt. Sta. Leaflet*, **7** (1925)
39. Sinclair, R. D., *Sci. Agr.*, **20**, 109–19 (1939)
40. Sheehy, E. J., and Senior, B. J., *J. Dept. Agr. (Ireland)*, **30**, 1–63 (1930)
41. Kirk, W. G., and Crown, R. M., *Florida Agr. Expt. Sta. Bull.*, **372** (1942)
42. Babcock, S. M., *Wisconsin Agr. Expt. Sta. Ann. Rept.*, **22**, 129–56 (1905)
43. Evvard, J. M., Brown, L. C., Culbertson, C. C., and Hammond, W. E., *Iowa Agr. Expt. Sta. Research Bull.*, **94** (1926)
44. Hudson, R. S., *Mich. Agr. Expt. Sta. Quart. Bull.*, **8**, 103–5 (1926)
45. Peck, E. F., *Vet. Record*, **50**, 409–10 (1938)
46. Hartman, A. M., *U.S. Dept. Agr. Yearbook*, 1027–44 (1939)
47. Griem, W. B., Hart, E. B., Kalkus, J. W., and Welch, H., "Second Report of the Committee on Animal Nutrition," *Natl. Research Council Circ.*, **111** (1942)
48. Evvard, J. M., and Culbertson, C. C., *Iowa Agr. Expt. Sta. Research Bull.*, **86** (1925)
49. Hopkirk, C. S. M., Dayus, C. V., Simpson, B. W., and Grimmett, R. E. R., *New Zealand J. Agr.*, **40**, 226–32 (1930)
50. Dayus, C. V., Taylor, B. A., and Thompson, G. A., *New Zealand J. Agr.*, **63**, 387–88 (1941)
51. Batt, F., *Norsk Vet. Tids.*, **52**, 89–98 (1940)
52. Malan, A. I., du Toit, P. J., and Groenewald, J. W., *Onderstepoort J. Vet. Sci. Animal Ind.*, **14**, 329–34 (1940)
53. Huffman, C. F., Duncan, C. W., Robinson, C. S., and Lamb, L. W., *Mich. Agr. Expt. Sta. Tech. Bull.*, **134** (1933)
54. Hirschfeld, E., *Queensland Agr. J.*, **54**, 196–207 (1940)
55. Duthie, D. W., *Agr. J. Brit. Guiana*, **10**, 194–204 (1939)
56. Montedonico, L. A., Fynn, C. A., and Vedani, F. O., *Rev. Facultad Agron. (Univ. Montevideo)*, **18**, 115–41 (1939)
57. Spangenberg, G. E., *Jornadas agron. vet. (Buenos Aires)*, *1939*, 9–37 (1940)
58. Fynn, C. A., *Rev. Facultad Agron. (Univ. Montevideo)*, **21**, 51–61 (1940)
59. Lecky, T. P., *J. Jamaica Agr. Soc.*, **43**, 418–24 (1939)
60. Menon, A. S., *Current Sci.*, **8**, 230–31 (1939)
61. Gomez, M. M., and Quevedo, J. M., *Rev. Argentina Agron.*, **8**, 77–78 (1941)
62. du Toit, P. J., Malan, A. I., van der Merwe, P. K., and Louw, J. G., *Farming in S. Africa*, **15**, 233–48 (1940)
63. du Toit, P. J., Louw, J. G., and Malan, A. I., *Onderstepoort J. Vet. Sci. Animal Ind.*, **14**, 123–327 (1940)

64. Ross, S. D., *Third West African Agr. Conf. 1938*, **1**, 427–90 (1940)
65. Bisschop, J. H. R., Malan, A. I., Steyn, H. P., and Laurence, G. B., *Onderstepoort J. Vet. Sci. Animal Ind.*, **13**, 321–43 (1939)
66. Ender, F., *Skand. Vet.-Tids.*, **32**, 378 (1942); cited in *Vet. Bull.*, **13**, 25 (1943)
67. Eckles, C. H., Becker, R. B., and Palmer, L. S., *Minnesota Agr. Expt. Sta. Bull.*, **229** (1926)
68. Fraps, G. S., and Cory, V. L., *Texas Agr. Expt. Sta. Bull.*, **586** (1940)
69. Black, W. H., Tash, L. H., Jones, J. M., and Kleberg, R. J., Jr., *U.S. Dept. Agr. Tech. Bull.*, **856** (1943)
70. Knox, J. H., Benner, J. W., and Watkins, W. E., *New Mex. Agr. Expt. Sta. Bull.*, **282** (1941)
71. Quinlan, J., Bisschop, J. H. R., and Adelaar, T. F., *Onderstepoort J. Vet. Sci. Animal Ind.*, **16**, 213–41 (1941)
72. Knox, J. H., and Watkins, W. E., *New Mex. Agr. Expt. Sta. Bull.*, **287** (1942)
73. Various Authors, *52nd Ann. Rept. Ariz. Agr. Expt. Sta.* (1941)
74. Underwood, E. J., Shier, F. L., and Beck, A. B., *J. Dept. Agr. W. Australia*, **17**, 388–405 (1940)
75. Beeson, W. M., Terrill, C. E., and Bolin, D. W., *J. Animal Sci.*, **2**, 369 (1943)
76. Hoflund, S., *North Am. Veterinarian*, **23**, 27–31 (1942)
77. Becker, R. B., Neal, W. M., and Shealy, A. L., *Florida Agr. Expt. Sta. Tech. Bull.*, **262** (1933)
78. Huffman, C. F., *J. Dairy Sci.*, **22**, 889–980 (1939)
79. Palmer, L. S., Fitch, C. P., Gullickson, T. W., and Boyd, W. L., *Cornell Vet.*, **25**, 229–46 (1935)
80. Vernon, H. A., *Vet. J.*, **88**, 512–15 (1932)
81. Jones, J. M., and Stangel, W. L., *Texas Agr. Expt. Sta. Bull.*, **563** (1938)
82. Rajagopalan, V. R., *Indian J. Vet. Sci.*, **9**, 415–24 (1939)
83. Wall, S., *Skand. Vet.-Tids.*, **25**, 169–204 (1935)
84. Greenlee, C. W., *Cornell Vet.*, **29**, 115–24 (1939)
85. Way, C., *J. Am. Vet. Med. Assoc.*, **100**, 335–39 (1942)
86. Witjens, J. C., *Nederland.-indische bl. diergeneeskunde*, **51**, 297–327 (1939)
87. Kernkamp, H. C. H., *Minnesota Agr. Expt. Sta. Tech. Bull.*, **31** (1925)
88. Aubel, C. E., Hughes, J. S., and Peterson, W. J., *J. Agr. Research*, **62**, 531–42 (1941)
89. Maynard, L. A., *Ann. Rev. Biochem.*, **10**, 449–70 (1941)
90. Maynard, L. A., and Loosli, J. K., *Ann. Rev. Biochem.*, **12**, 251–72 (1943)
91. McNaught, K. J., and Paul, G. W., *New Zealand J. Sci. Tech.*, **21B**, 95–101 (1939)
92. McNaught, K. J., and Paul, G. W., *New Zealand J. Sci. Tech.*, **21A**, 343–44 (1940)
93. Philp, R. C. T., Dumaresq, J. A., and Wilson, R. J., *Tasmanian J. Agr.*, **11**, 187–92 (1940)
94. Philp, R. C. T., and Wherrett, A. B., *Tasmanian J. Agr.*, **13**, 22–23 (1942)
95. McDonald, I. W., *Australian Vet. J.*, **18**, 107–15 (1942)

96. STEWART, J., MITCHELL, R. L., AND STEWART, A. B., *Empire J. Exptl. Agr.*, **10**, 57–60 (1942)
97. DUNLOP, G., AND MCCALLIEN, W. J., *Nature*, **147**, 615–17 (1941)
98. BOWSTEAD, J. E., SACKVILLE, J. P., AND SINCLAIR, R. D., *Sci. Agr.*, **22**, 314–25 (1942)
99. BOWSTEAD, J. E. SACKVILLE, J. P., AND SINCLAIR, R. D., *Sci. Agr.*, **22**, 479–81 (1942)
100. BALTZER, A. C., KILLHAM, B. J., DUNCAN, C. W., AND HUFFMAN, C. F., *Mich. Agr. Expt. Sta. Quart. Bull.*, **24**, 68–70 (1941)
101. BENNETTS, H. W., BECK, A. B., HARLEY, R., AND EVANS, S. T., *Australian Vet. J.*, **17**, 85–93 (1941)
102. BENNETTS, H. W., HARLEY, R., AND EVANS, S. T., *Australian Vet. J.*, **18**, 50–63 (1942)
103. BECK, A. B., *J. Dept. Agr. W. Australia*, **18**, 285–300 (1941)
104. MAHAFFEY, L. W., BENNETTS, H. W., AND FLOOD, A. F., *Australian Vet. J.*, **18**, 205–8 (1942)
105. VARIOUS AUTHORS, *J. Dept. Agr. S. Australia*, **44**, 299–303 (1941)
106. BENNETTS, H. W., *J. Dept. Agr. W. Australia*, **19**, 7–13 (1942)
107. INNES, J. R. M., AND SHEARER, G. D., *J. Comp. Path. Therap.*, **53**, 1–41 (1940)
108. TABUSSO, M. E., *Pub. Inst. Nacion. Biol. Animal*, **1**, 1–38 (Lima, Peru, 1942)
109. DU TOIT, J. P., *Farming in S. Africa*, **14**, 511–19 (1939)
110. OLAFSON, P., *Cornell Vet.*, **32**, 301–14 (1942)
111. NEAL, W. M., *103rd Meeting Am. Chem. Soc., Abstract Papers*, 7A (1942)
112. BROUWER, E., FRENS, A. M., REITSMA, P., AND KALISVAART, C., *Verslag. Landb. Onderzoek., C.*, **44**, 267–98 (1938)
113. FRENS, A. M., *Tijdschr. Diergeneesk.*, **68**, 763–68 (1941)
114. SCHEY, L. T. C., AND KALISVAART, C., *Landbouwkund. Tijdschr.*, **50**, 598–619 (1938)
115. THIJN, J. W., *Tijdschr. Diergeneesk.*, **67**, 421–46 (1940)
116. GEERTSEMA, G., *Tijdschr. Diergeneesk.*, **67**, 176–85 (1940)
117. VARIOUS AUTHORS, *Vet. Med.*, **35**, 357–58 (1940)
118. ARCHIBALD, J. G., KUCINSKI, K. J., BROOKE, R. O., AND FREEMAN, S. L., *J. Dairy Sci.*, **21**, 59–68 (1938)
119. ARCHIBALD, J. G. (Personal communication)
120. SVANBERG, O., HANNERZ, E., AND WIJKSTRÖM, T., *Lantbruks-Högskol. Ann.*, **2**, 31–50 (1935)
121. FOOT, A. S., AND THOMSON, S. Y., *J. Ministry Agr.* (*Engl.*), **45**, 452–59 (1938)
122. SWALES, W. E., CRAMPTON, E. W., ASHTON, G. C., AND CHOQUETTE, L., *Can. J. Research*, **20**, 380–86 (1942)
123. ARCHIBALD, R. M., AND HANCOCK, E. E. I., *Can. J. Comp. Med. Vet. Sci.*, **3**, 134 (1939)
124. MUSSIL, J., *Wien. tierärtzl. Monschr.*, **28**, 136–37 (1941)
125. HART, G. H., AND GUILBERT, H. R., *Calif. Agr. Expt. Sta. Bull.*, **560** (1933)
126. HUGHES, J. S., *North Am. Veterinarian*, **17**, (3), 22–26 (1936)
127. HART, G. H., *Nutrition Abstracts & Revs.*, **10**, 261–72 (1940–41)

128. Snyder, W. W., and Moore, L. A., *J. Dairy Sci.*, **23**, 363–71 (1940)
129. Converse, H. T., and Meigs, E. B., *Proc. Am. Soc. Animal Production*, **24**, 141–44 (1932)
130. Phillips, P. H., Lundquist, N. S., and Boyer, P. D., *J. Dairy Sci.*, **24**, 977–82 (1941)
131. Stewart, J., and McCallum, J. W., *J. Comp. Path. Therap.*, **51**, 290–95 (1938)
132. Henry, K. M., Houston, J., and Kon, S. K., *J. Dairy Research*, **11**, 1–8 (1940)
133. Stewart, J., and McCallum, J. W., *J. Dairy Research*, **13**, 1–4 (1942)
134. Kramer, M. M., Bair, M. D., Kunerth, B. L., and Riddell, W. H., *J. Agr. Research*, **56**, 227–32 (1938)
135. Benham, G. H., *Can. J. Comp. Med. Vet. Sci.*, **7**, 291–97 (1943)
136. Cady, J., *Vet. Med.*, **37**, 223–24 (1942)
137. Sampson, J., Boley, L. E., and Graham, R., *Cornell Vet.*, **28**, 53–57 (1938)
138. Sánchez, F., *Berl. tierärtzl. Wochschr.*, **49**, 792–94 (1933)
139. Schieblich, M., *Berl. tierärtzl. Wochschr.*, **50**, 338–39 (1934)
140. Various Authors, *Ann. Rept. Imperial Vet. Research Inst. (Mukteswar), 1937–38* (1940)
141. Fernandes, C. J., *Indian Farming*, **1**, 591–92 (1940)
142. Rose, V. T., *J. Am. Vet. Med. Assoc.*, **100**, 234–36 (1942)
143. Gunn, R. M. C., *Australian Vet. J.*, **18**, 94–106 (1942)
144. Immenschuh, R. D., *Vet. Med.*, **36**, 322–23 (1941)
145. Beeson, W. M., Pence, J. W., and Holm, G. C., *Am. J. Vet. Research*, **4**, 120–26 (1943)
146. Klemola, V., *Biedermanns Zentr. B. Tierernähr.*, **5**, 657–75 (1933)
147. Chatelain, M. P., *Bull. Soc. Sci. vet.* (*Lyon*), **36**, 223–36 (1933)
148. Meadows, D., *Vet. J.*, **75**, 140–41 (1919)
149. Kesler, R. A., and Callender, G. R., *Vet. Med.*, **33**, 307–20 (1938)
150. Hart, G. H., Goss, H., and Guilbert, H. R., *Am. J. Vet. Research*, **4**, 162–68 (1943)
151. Foot, A. S., Henry, K. M., Kon, S. K., and Mackintosh, J., *J. Agr. Sci.*, **29**, 142–63 (1939)
152. Golîarkin, F. E., *Proc. Lenin Acad Agr. Sci.* (*U.S.S.R.*), **9**, 33–36 (1941)
153. Albright, W. D., and Fraser, L. D., *Dept. Agr.* (*Canada*) *Publ.*, **736** (1942)
154. Hunt, C. H., Burroughs, E. W., Bethke, R. M., Schalk, A. F., and Gerlaugh, P., *J. Nutrition*, **25**, 207–16 (1943)
155. Lundquist, N. S., and Phillips, P. H., *J. Dairy Sci.*, **26**, 1023–30 (1943)
156. Reid, J. T., Huffman, C. F., and Duncan, C. W., *J. Dairy Sci.*, **26**, 738–39 (1943)
157. Dynna, O., and Thune, I., *Norsk. Vet. Tids.*, **53**, 94–96 (1941)
158. Pallaske, G., *Arch. wiss. prakt. Tierheilk.*, **70**, 278–91 (1936)
159. Madison, L. C., Miller, R. C., and Keith, T. B., *Science*, **89**, 490–91 (1939)
160. Davis, G. K., Freeman, V. A., and Madsen, L. L., *Mich. Agr. Expt. Sta. Tech. Bull.*, **170** (1940)
161. Chernyshev., G. V., *Sovyet Vet.*, **12**, 57–58 (1939)

162. Wallis, G. C., *J. Dairy Sci.*, **26**, 401–8 (1943)
163. Phillips, P. H., Rupel, I. W., Oleson, J. J., and Bohstedt, G., *Proc. Am. Soc. Animal Production*, **31**, 320–27 (1938)
164. Phillips, P. H., Lardy, H. A., Boyer, P. D., and Werner, G. M., *J. Dairy Sci.*, **24**, 153–58 (1941)
165. Knight, C. A., Dutcher, R. A., Guerrant, N. B., and Bechdel, S. I., *J. Dairy Sci.*, **24**, 567–77 (1941)
166. Sutton, T. S., Kaeser, H. E., and Hansard, S. L., *J. Biol. Chem.*, **144**, 183–91 (1942)
167. Boyer, P. D., Phillips, P. H., Pounden, W. D., Jensen, C. W., Rupel, I. W., and Nesbit, M. E., *J. Nutrition*, **23**, 525–31 (1942)
168. Bortree, A. L., Huffman, C. F., and Duncan, C. W., *J. Dairy Sci.*, **26**, 553–62 (1943)
169. Scheidenhelm, E. C., Bortree, A. L., Huffman, C. F., and Clark, C. F., *J. Dairy Sci.*, **25**, 690–91 (1942)
170. Satterfield, G. H., Bailey, E. A., Jr., Foster, J. E., and Hostetler, E. H., *J. Nutrition*, **24**, 121–29 (1942)
171. Shchepetov, F. N., and Ovsyannikova, A. V., *Veterinariya (Moscow)*, **3**, 130–33 (1941)
172. Davis, G. K., and Cole, C. L., *J. Animal Sci.*, **2**, 53–58 (1943)
173. Duncan, C. W., and Huffman, C. F., *J. Dairy Sci.*, **19**, 291–303 (1936)
174. Olson, T. M., *S. Dakota Agr. Expt. Sta. Bull.*, **319** (1938)
175. Bechtel, H. E., and Hoppert, C. A., *Mich. Agr. Expt. Sta. Quart. Bull.*, **18**, 153–54 (1936)
176. Bechtel, H. E., Huffman, C. F., Duncan, C. W., and Hoppert, C. A., *J. Dairy Sci.*, **19**, 359–72 (1936)
177. Johnson, D. W., and Palmer, L. S., *J. Agr. Research*, **63**, 639–48 (1941)
178. Rupel, I. W., Bohstedt, G., and Hart, E. B., *Wisconsin Agr. Expt. Sta. Research Bull.*, **115** (1933)
179. Gullickson, T. W., Palmer, L. S., and Boyd, W. L., *Minnesota Agr. Expt. Sta. Tech. Bull.*, **105** (1935)
180. Fitch, L. W. N., *Australian Vet. J.*, **19**, 2–20 (1943)
181. Hastings, C. C., *North Am. Veterinarian*, **23**, 650 (1942)
182. Moussu, R., *Rev. Méd. vét.*, **111**, 905–19 (1935)
183. Lentz, R. W., *Berl. tierärtzl. Wochschr.*, Nr. **14**, 201–2 (1938)
184. Vogt-Möller, P., and Bay, F., *Vet. J.*, **87**, 165–70 (1931)
185. Bay, F., and Vogt-Möller, P., *Vet. J.*, **90**, 288–90 (1934)
186. Thomas, B. H., Cannon, C. Y., McNutt, S. H., and Underbjerg, G., *J. Nutrition, Suppl.*, **15**, 10 (1938)

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THE BIOCHEMISTRY OF MALIGNANT TISSUE

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The first two chapters on cancer in the *Annual Review of Biochemistry* appeared in 1934 (1) and 1935 (2) and reported a total of 150 papers for a five year period. The next chapter, in 1940 (3), treated 250 references. Since then, over 2000 original papers bearing directly on the biochemistry of cancer have been published. Thus, in spite of wartime restrictions on output in many fields of scientific investigation, research on cancer has continued at not only undiminished but increasing pace, and now ramifies into every aspect of biochemistry. Obviously, the present chapter must be limited to a highly restricted survey. It will report on tumor constituents, metabolism, carcinogenesis, and chemotherapy. On grounds of space limitation alone, the following equally important biochemical aspects of cancer, each of which might warrant a separate chapter, have arbitrarily been eliminated: nutrition, mainly vitamins, and just reviewed by us elsewhere (4); hormones, just reviewed (5); viruses (macromolecular particles); immunology; and diagnosis (experimental and clinical); as well as many hundreds of references on purely biological aspects (growth response, morphology, and pathology) or purely chemical (synthetic) aspects of carcinogenesis. Hartwell's survey of carcinogenic compounds (6), published in 1941, lists some 1200 references, and since then about 600 more references on carcinogenesis by known chemical compounds have appeared, apart from carcinogenesis by chemical mixtures and other means. The new book by Stern & Willheim (7) contains some 5000 references which cover both recent material and probably the best summary of biochemical work on cancer between 1900 and 1930, especially foreign works, and is unusually valuable on this latter account. The shorter book by von Euler & Skarzynski (8) is the more strictly biochemical. Two large and representative Russian books on oncology, written by groups of collaborators, have been abstracted in *Cancer Research* (9, 10). Reviews on special aspects of cancer biochemistry may be consulted (5, 11 to 22, and others indicated later).

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TUMOR CONSTITUENTS

Vitamins.—Several reviews on this subject have appeared recently (4, 7, 23). The suggestion that cancer tissues might have a higher content or requirement and concentration of certain of the essential growth factors has been advanced repeatedly. However, this has not proved a valid generalization in many cases of homologous normal and cancerous tissue pairs studied in recent years, with respect to riboflavin, niacin, biotin, pantothenic acid, folic acid, inositol, thiamin, and pyridoxin (24 to 29). The concentration of each of these B-complex vitamins was much more uniform among different tumors than in the tissues from which the tumors were derived (30). This can be interpreted as further evidence that cancers are de- or less differentiated than homologous adult tissues to the extent of forming a group of tissues of rather uniform biochemical type, as has long been indicated by overall metabolism studies on respiration and glycolysis.

It is often the case that the vitamin content of a tumor deviates from that in homologous, normal adult tissue in the same direction as corresponding embryonic tissue, as best shown so far for biotin (31, 32). Liver, as a storage organ, usually contains a much greater vitamin content than hepatomas, especially in the case of biotin (31, 32), and riboflavin (25, 33 to 36), with the exception of mouse hepatomas (35, 38). A high content of riboflavin has been reported for certain human carcinomas (37). The avidin uncombinable fraction of the total biotin vitamer (38) content of tumors is usually quite high, both absolutely, and relatively, when compared with that in homologous normal tissues (39, 40, 41), and is suggestive of abnormal biotin vitamer metabolism in tumors.

The content of coenzymes I and II was found to be low in leukemic cells (42). Leukemic white cells had several fold higher total thiamin level than normal white cells (43) but about the same thiamin/cocarboxylase ratio as normal white cells (44). Enzyme systems utilizing cocarboxylase were not found in either normal or leukemic white cells (44). Leukemic erythrocytes contained twice the normal thiamin content (43).

Robertson (45) has made an extensive comparison between the ascorbic acid contents of homologous normal and tumor tissues and found no evident relationship between the ascorbic acid content and the rate of growth or the ascorbic acid level of the tissue of origin. Of tumors investigated, seven were lower in ascorbic acid content,

nine were higher, and four were the same as the tissues of origin. Comparative dehydroascorbic acid and bound ascorbic acid values of tumors and normal tissues have been reported (46). Leukemic white cells had a high concentration of ascorbic acid or similar reducing substance, as compared with normal white cells and in relation to plasma values (47).

Hepatomas induced in rats with *p*-dimethylaminoazobenzene yielded cholesterol crystals, but contained no provitamin D, as did neither noncancerous or precancerous liver; Iikubo transplantable hepatoma contained 7 per cent provitamin D (48).

The many reports that the vitamin A content of the livers or liver mitochondria of rats, mice, or rabbits is markedly reduced by injection of certain carcinogenic compounds (49 to 59), and the fact that hepatomas induced by feeding rats 2-amino,5-azotoluene, or other hepatomas (60), contain little or no vitamin A (52), suggest that vitamin A might be definitely concerned in tumor genesis here, and might be generally lacking in tumors. However, it has been reported, upon the basis of fluorescence microscopy, that tumors usually contain vitamin A if they arise from tissues containing appreciable amounts of the vitamin (61). The presence and distribution of vitamin A fluorescence aided in determining tumor origin in instances. The vitamin A content of exudate cells from Erlich mouse sarcoma ascites has been compared with nonmalignant exudate cells by histochemical staining reactions, and apparently decreased as the degree of malignancy of the cells increased (62).

The vitamin E content of Jensen rat sarcoma and Brown-Pearce rabbit sarcoma has been reported to be higher than in many normal tissues (ca. 4 mg. α-tocopherol per 100 gm. wet weight), whereas fat soluble carotenoids were not found in the tumors (63, 64).

The choline and acetyl choline content of a number of human tumors of the central nervous system were determined by the leech muscle method and were found to be of the same order of magnitude as in normal nerve tissue (65).

Enzymes. — Several recent reviews have appeared (11, 14). Greenstein and co-workers have made extensive studies of a number of homologous normal and tumor tissues, particularly hepatic ones, with respect to several enzymes (arginase, amylase, xanthine dehydrogenase, catalase, acid phosphatase, alkaline phosphatase, thymodepolymerase, ribodepolymerase), and have shown that whether the content of a given enzyme is relatively increased or decreased in a

tumor depends upon a number of factors, including type of tumor and enzyme, and species and strain of animal (66 to 76). Among hepatic tissues, the enzyme makeup of fetal rat liver more closely resembled that of malignant rat hepatoma than that of normal rat liver. The activities of various enzymes were much more uniform in mouse tumors than in normal mouse tissues (76), a situation paralleling the distribution of the B-complex vitamins already mentioned (30) and again suggesting that tumors are of a de- or less differentiated, common biochemical tissue type than their normal homologues.

Greenstein and co-workers have demonstrated that the presence of a tumor may influence the enzymatic activities of host tissues remote from the site of the tumor (11). The outstanding example of this effect has been the reduction of liver catalase activity in rats and mice bearing tumors (67, 72, 77 to 80). The mechanism of this systemic effect of tumors on liver catalase has not been worked out. Embryonic mouse tissue implanted subcutaneously in mice grew for several months, but caused no decrease in liver catalase (81). No direct action of tumor extracts on the activity of liver catalase was evident *in vitro*. It has been suggested that the effect may be the result of interference with the synthesis of the hematoporphyrin nucleus for both catalase and hemoglobin (82). It would be interesting to know how liver catalase is affected by other conditions such as burns, trauma, and infections since this might indicate how specific the effect is for cancer. In livers of tumor-bearing rats arginase has also been observed to decrease (71), the water content (35, 83) and copper content (84) to increase, and the acid and alkaline phosphatase (66), xanthine dehydrogenase (67), amylase (67), ribose- and desoxyribosenucleic acid depolymerases (67, 74), cytochrome oxidase (85), and peptidase (86) not to change. The *d*-amino acid oxidase activity was often reduced to 30 to 50 per cent of normal in livers of tumor-bearing rats (87 to 92), as compared to values about one tenth normal in hepatomas (93). Decreases have been noted in the *d*-amino acid oxidase apoenzyme, uricase, and choline oxidase in livers of rats bearing hepatoma 31 (but not Walker carcinosarcoma 256), and in the apoenzyme and uricase of liver nuclei; the hepatoma 31 and nuclei thereof contained only 3 to 5 per cent of the apoenzyme and uricase activity of normal liver, and no choline oxidase, and the tumor 256 contained none of these three oxidases (94, 95).

Differences in susceptibility of various strains of mice to spontaneous or induced cancer may find some basis in enzymic differences

in certain tissues and organs. The xanthine dehydrogenase activity of livers of the cancer-susceptible strain C3H mice was about half that of the cancer-resistant strain JK mice, suggesting differences in nucleoprotein or aldehyde metabolism between these strains (96). The activity of serum butyric esterase has been reported to be about twice as high in two strains of cancer-susceptible mice (strains A and C3H) as in a cancer-resistant strain (C57 black) (97); this has been correlated with the excretion of the enzyme rather than with its production in the liver, since the fecal excretion of esterase was higher in the resistant mice, whereas the liver (and kidney) esterase was not significantly different (98). Reciprocal foster-nursing altered the serum esterase content; it was decreased by nursing with low mammary cancer mothers, and vice versa, but liver esterase was unaffected (99). The hemoglobin level of strains of mice of high cancer incidence has been noted to fall off markedly before cancers appeared, in contrast to control resistant strains (93). Mice of high cancer incidence have also been reported to have larger amounts of fluorescent porphyrins in the Harderian glands than do mice of resistant strains (100, 101).

Earlier observations (102, 103, 104) that tumors usually have less cytochrome-*c* and cytochrome oxidase than normal tissues seem to be fairly well supported by more recent work (85, 105, 106, 107), and in accord with observations that normal tissue slices show a greater capacity to increase their respiration upon addition of *p*-phenylenediamine and succinate than do tumor tissue slices (40, 41, 108, 109). Albaum & Potter (110) and Elliott (111) have made further studies on naturally occurring inhibitors of succinoxidase preparations obtained mainly from tumors but also in instances from normal tissues.

There appears to be a direct relationship between tumor phosphatase activity and phosphate deposition since it has been shown that the rate of deposition of radioactive phosphate in bone tumors is proportional to the alkaline phosphatase activity of the tumors (112). Histochemical determinations of the distribution of alkaline phosphatase in various normal and neoplastic tissues have been made by observing the deposition of calcium at the site of phosphatase action when tissues are incubated with sodium β-glycerophosphate; the alkaline phosphatases often, though not always, appeared to be most concentrated in tissues concerned in calcium deposition or in glucose absorption or in tumors derived from such tissues (113 to 118). The alkaline phosphatase activity of rat hepatomas induced by feeding *p*-dimethylaminoazobenzene has been shown by several investigators to be higher

than in normal liver (66, 115, 118, 119). This is in agreement with observations that calcium deposition sometimes occurs in this type of tumor (118), and may represent an attempt at compensation for inhibition of some dephosphorylation mechanisms by *p*-dimethylaminoazobenzene or its derivatives (119). Weil (120) has noted that rats with Jensen sarcoma did not show any change in plasma phosphatase during tumor growth or regression.

Warburg & Christian (121) reported that the plasma of rats bearing Jensen sarcoma contains several times as much zymohexase as normal rat plasma. Kubowitz & Ott (122) have isolated from Jensen rat sarcoma an enzyme acting on phosphopyruvic acid to form lactic acid.

Pirie (123) has reported further work on the spreading factor, or hyaluronidase activity, in several mammalian tumors and fowl fibromas.

Earlier observations on transaminase activity (124) in tumors have been extended to rat hepatomas and liver (125) and to mouse tumors and fetal, kitten, and adult cat tissues (126). Most tumors and rapidly growing tissues, including regenerating liver, appear to have a low transaminase activity. This has given rise to the suggestion that protein synthesis is faster when transaminase activity is low, since the activity of this enzyme could eliminate glutamic acid necessary therefor.

Decreases in the concentration of coenzymes I and II in malignant tissue (33, 42, 127) have been observed.

Maver and co-workers (86, 128, 129) have studied the proteinase and peptidase activities of extracts of rat hepatoma and normal and regenerating rat liver. It was found that the hepatoma contained the most proteinase activity (hemoglobin hydrolysis) and peptidase activity (*d,l*-leucylglycine hydrolysis) of the three tissue types, indicating a definite alteration in protein metabolism as the liver became neoplastic. The peptidase activity of Jensen rat sarcoma compared with rat liver, kidney, and spleen disclosed qualitative differences in capacity to hydrolyze different synthetic peptides. Similar differences in autolytic capacity between tumors and normal tissues have been reported (130, 131). Immunological differences and similarities in purified cathepsins from normal rat liver, transplanted hepatoma 31, and Jensen sarcoma have been demonstrated by serological and anaphylactic techniques (132), and indicate that the cathepsins may be somewhat altered as tissues become cancerous.

The report that the sera of patients with carcinoma could hydrolyze certain peptidases containing unnatural amino acids (133) has stimulated a great many similar studies involving normal serum and serum from cancer patients and animals as well as extracts of normal and cancerous tissues (133 to 165). Results of these studies have been exceedingly variable and inconsistent, and while it is certain that *d*-peptidase activity does sometimes occur in animal tissues, it is quite clear that *d*-peptidase activity is not a specific characteristic of malignant tumors. It is probable that many of the cases of the *d*-peptidase activity observed are to be ascribed to unnoted microbiological contaminations; at any rate most of the literature cited, which involves hydrolyses lasting one or several days at 30 to 37° C., must be read with this factor in mind. *d*-Peptidase activity may be more related to the age of animal than to the occurrence of malignant tissue (157).

Unnatural amino acids.—In spite of some thirty papers to the contrary, Kögl and collaborators (166, 167) still continue to support vigorously their claim that the proteins of cancer tissue are characterized by the presence of amino acids of unnatural configuration, especially *d*-glutamic acid (168). After an interim of three years since their previous experimental paper of the series, these authors, employing deuterium analysis (166), report the presence of 20 to 24 per cent *d*-glutamic acid in the total glutamic acid in various Brown-Pearce tumor metastases, 21 to 22 per cent in human tumor metastases, 11 to 14 per cent in chemically induced tumors, and 9 per cent in Flexner-Jobling carcinoma transplants, as compared with 2 per cent in benign uterine tumors, and less than 1 per cent in normal or embryonic tissues. The tissue protein of all malignant tumors analyzed appeared to contain 1 to 4 per cent *d*-glutamic acid, as compared with 0.1 per cent or less in all normal or embryonic tissue proteins. These results are in close agreement with earlier results obtained by Kögl by isolation.

A prominent feature of the new data is the high content (13 to 15 per cent) of *l*-glutamic acid claimed for all tissue proteins examined, malignant or nonmalignant. These high values were obtained as a result of hydrolysis for twenty hours instead of seven hours as employed formerly, whereby values only one third as great were obtained with normal tissues and one half to three quarters as great with malignant tissues. The high twenty-hour values for *l*-glutamic acid are much higher than any values obtained by workers previously by either isolation or analysis after seven to twenty-two hours' hydrolysis, and

this suggests to Kögl the possibility of some systematic error in determinations by others of not only *l*- but also of *d*-glutamic acid. However, Kögl found no appreciable increases in *d*-glutamic acid after twenty as compared with seven hours' hydrolysis of either normal or malignant tissue, so that it is not likely that time of hydrolysis was an important factor in the earlier analyses by others for *d*-glutamic acid by means of isotopes (169) or *d*-amino acid oxidase (170, 171). With both of these methods Kögl claimed difficulty in obtaining recovery of *d*-glutamic acid added to hydrolyzates (166, 172), but this reported lack of success is not to be regarded as a reflection on either of the methods, which in the case of the *d*-amino acid oxidase is simple, direct, and conclusive, even in the matter of recoveries.

Kögl states that the object of his new and arresting work is to reinforce his earlier isolation work by isotopic (deuterium) analyses, and he elects not to provide any satisfactory explanation for the many opposing results obtained by others, who, however, have good reason to believe that the bulk of their results and final conclusions are as acceptable as his, to say the least. Kögl makes the plea that, in a mutual search for truth by opposing sides of the *d*-glutamic acid question, more weight be given to the "positive" results obtained by him than to the "negative" results of others. He overlooks the fact, however, that the results of others are in reality just as positive as his, merely opposed thereto; for his conclusion is the negative of the positive conclusion reached by so many others, namely, that malignant and normal tissues have essentially the same amino acid makeup with respect to configuration.

The most recent works opposed to Kögl's conclusion are the two extensive investigations of Wieland (173) and Klingmüller (174), the latter appearing shortly after Kögl's latest paper (166); Abderhalden (175) reports variable results. Highly significant is the statement of Warburg (121) that the reported "stereochemical abnormality in tumor protein is an experimental error." In the judgment of the reviewers, there is perhaps one chance in twenty that Kögl's vigorously renewed claim (166) is correct, even for some tumors.

Other constituents.—Nucleoproteins isolated from animal livers, hepatomas, and Jensen rat sarcoma (176, 177, 178) have close similarity in amino acid and desoxyribonucleic acid composition (179). The minimum molecular weight of the tumor nucleoprotein calculated from the amino acid composition was 40,000. pH titration curves in 6 *M* guanidine hydrochloride indicated that the nucleoprotein was

capable of binding considerably more acid and base than beef serum albumin (177).

Almost all of the protein in a malignant mouse melanoma was water soluble and precipitated by 40 per cent ammonium sulfate, which suggested that it was a pseudoglobulin (180). The minimal molecular weight calculated from the content of several amino acids was about 20,000. The melanin pigment was attached to the protein and could be partially released by digestion with pancreatin, to yield a black residue that was very rich in sulfur-containing amino acids. This suggests that the pigment is attached to the protein through cystine or methionine.

Determinations of "free" and "total" cystine in aqueous extracts of rat and mouse livers and hepatomas (181) gave values for "free" cystine that correspond to the glutathione reported in these tissues by earlier workers. Rat hepatoma had significantly less "free" cystine than rat liver. More sulfhydryl groups were liberated from rat liver than from rat hepatomas upon the addition of denaturing salts.

Studies on the chemical constitution of lipids of mitochondria from normal and tumor cells have indicated an exceptionally high lipid content in the case of Jensen sarcoma (182).

A polysaccharide isolated from a virus-produced chicken tumor appeared to be similar to or identical with the polysaccharide found in vitreous humor, umbilical cord, and synovial fluid (123, 183). Slowly growing transplants of Walker rat tumor 256 contained more glycogen than faster growing transplants (184), and this relationship was not affected by hypophysectomy. The glycogen content of rat hepatomas induced by feeding *p*-dimethylaminoazobenzene was considerably less than in normal rat liver (118, 185).

The citric acid content of tumors has been found to be higher than most normal tissues except bone, seminal vesicles, and embryonic tissues (186, 187). The available evidence indicates that citrate may be important in calcium as well as carbohydrate metabolism.

The work of Lasnitzki & Brewer on the distribution of potassium isotopes in tissues continues to indicate that in most normal tissues the isotopic ratio K^{39}/K^{41} is the same (or less) than that observed in ordinary potassium salts, whereas the proportion of K^{41} may be significantly increased in tumors and tissues of tumor-bearing animals and humans (188 to 192). Changes in the proportion of the radioactive isotope K^{40} in tumor tissues were not detected (193).

Beltrami has reported that the ash content of induced tumors in-

creased with repeated transplantation (194). Kahler & Robertson (195) have reported on the intercellular hydrogen ion concentration in tumors and tissues, measured with special glass electrodes.

Metabolism

Glycolysis and respiration.—Several recent reviews have appeared on this subject (12, 196, 197). The central problem regarding tissue metabolism for the period under review has been the comparison of homologous normal, precancerous, benign, and malignant tissues with respect to hepatomas (185, 198 to 204), epithelial tumors (41, 109, 185, 201, 202, 205 to 209), sarcomas (40, 41, 109, 202, 210, 211, 212), leukemias (109, 202, 213 to 222), and chicken leukosis (213). Probably the most important single paper is that of Dickens & Weil-Malherbe (185), which shows that in the conversion of normal hepatic cells to malignant hepatoma there is almost total loss of certain highly specialized functions, including synthesis of fermentable carbohydrate from pyruvic acid, formation of acetoacetic acid from caprylic acid, and nitrogenous reactions to be listed in the next section. This paper and those by Kidd *et al.* (41) and Burk (197) bring up to date the lively controversy on the specificity of low respiratory quotient and the aerobic and anaerobic glycolyses for tumor metabolism (196, 205, 206, 207, 220, 223 to 228). It is quite clear that when enough criteria, including derived quotients, are taken into account (197, p. 242), between 95 and 100 per cent of all tumors or tumor types may be metabolically distinguished from nonneoplastic tissues including growing and embryonic ones. In regard to the apparently critical case brought up by Berenblum, Chain & Heatley (205, 206, 227), in which it was reported that normal skin epithelium of rabbit and Shope virus papilloma possessed essentially the same values for respiration and aerobic and anaerobic glycolyses, it may be pointed out that this was a comparison merely between a benign tumor and normal epithelium admixed with a considerable and unspecified amount of nonepithelial tissue. Kidd, Winzler & Burk (41) have now made a comparison between the benign Shope virus papilloma and the malignant, transplantable V_2 carcinoma derived therefrom, and have shown characteristic differences with respect to glycolytic capacity and derived metabolic quotients, and also certain differences between the papilloma and normal rabbit skin. It was the opinion of these workers that no histologically adequate tissue slice samples of epithelial tissue have been prepared from skin by any workers,

that were suitable for making any very exact metabolic comparisons between normal epidermal cells and benign or malignant tumor cells derived therefrom.

Other aspects of tissue metabolism studied have been respiration increases caused by certain indicators (229), effect of various inhibitors (211, 230, 231, 232), effect of temperature (233, 234), effect of vitamin A (235), effect of induced tumor resistance, and effect of methylcholanthrene on respiratory values of mouse skin prior to tumor formation (236, 237). Many studies have been specifically directed to ascertaining precancerous metabolic changes, with little positive result, except comparatively small effects, as in the case of precancerous livers (185, 197 to 200).

The Pasteur effect (196) has received further elucidation (209, 238, 239), especially at the hands of Engelhardt & Sarkov (240) who have further localized the Pasteur effect shunt (196, p. 451) as occurring at the Neuberg-ester-phosphorylase, whereby fructose-6-phosphate (Neuberg ester) is converted into fructose-1,6-diphosphate (Harden-Young ester) by adenosinetriphosphate. The prevailing oxidation-reduction potential determines whether phosphorylation of the Neuberg ester followed by fermentation takes place on the one hand or oxidation through phosphogluconic acid and similar stages on the other. The role of oxygen pressure has continued to receive attention in tissue metabolism studies (218, 219, 221, 239), and the influence of oxygen pressure on tumors *in vivo* has also been studied (241 to 244).

Tissue culture metabolism.—Studies on tissue culture tumor metabolism or growth have been reported with respect to methodology (245 to 248), and to the effects of *p*-dimethylaminoazobenzene (249, 250), amines (251), nicotine (252), gibberellin (253), polysaccharide (254), colchicine (255), macromolecular material (256), radium (257), x-rays (258, 259, 260) and low temperature (261). The metabolism of mouse sarcomas derived from tissue cultures of normal mouse fibroblasts (262) has been found to be characteristic of the metabolism of malignant tumors generally (40).

Nitrogen metabolism.—The transformation of normal liver to malignant hepatomas in rats is accompanied by changes in nitrogen metabolism that are as profound as those observed in carbohydrate metabolism. Thus transaminase (125), *d*-amino acid oxidase (85), xanthine dehydrogenase (70), oxidation of uric acid (185), degradation of cystine (263), and formation of urea from ammonia or

alanine (185) or from ammonia and citrulline (264), have been shown to be markedly reduced in primary or transplanted rat hepatomas induced by feeding *p*-dimethylaminoazobenzene. The activity of some of the proteolytic enzymes may remain unchanged or may increase (128). The observation that the creatine content is the same in normal liver and in hepatoma suggests that creatine metabolism is not altered in the transformation of liver to malignant tissue (265).

Radiophosphate metabolism—nucleoproteins and phospholipids.—Considerable use has been made of radioactive phosphorus, P^{32}, for studying phosphate, nucleoprotein, and phospholipid metabolism of normal and malignant tissues of patients and experimental animals (266 to 289). These studies have indicated that malignant tissues take up radiophosphate more readily and release it more slowly than do most other tissues, except liver and small intestine. It appears that the nucleoprotein and phospholipid fractions are chiefly responsible for the uptake and retention of P^{32}. Marshak (56, 290) has isolated the nuclei from liver and tumor tissues and shown that most of the P^{32} taken up by these cells in short periods was in the nucleoprotein fractions. Tumor nuclei took up more P^{32} than did liver nuclei. The breakdown of adenylic acid, nucleic acid, and related compounds to hypoxanthine by dialyzed Jensen rat sarcoma extracts in the presence of a hydrogen acceptor has been demonstrated (291). This destruction is in agreement with an observation that the uric acid content of tumors was generally higher than in normal tissues (85, 292), and suggests an alteration in the metabolism of nucleotides and nucleosides in tumor tissue.

The use of P^{32} in tracer and therapeutic doses for acute and chronic leukemia patients and other cancer patients has now been reported by a number of workers (30, 270, 271, 275 to 278, 280, 281, 285 to 288, 293 to 298). It seems established that the differential accumulation of P^{32} in leukocytes and malignant tissues gives a distribution that is frequently favorable to the selective radiation of the malignant cells. Evidence that the administration of subtherapeutic amounts of radioactive phosphate to leukemic patients leads to an increase in organic acid-soluble phosphorus of the blood cells has suggested that even tracer amounts of P^{32} may alter phosphorus metabolism (299). The mechanism of the arresting action of P^{32} on cancer is still uncertain since Sugiura (300) found that the transplantability of mouse sarcoma 180 was diminished only

by lengthy exposure to solutions of relatively high P^{32} content (ca. 100 micro curies per cc. for 24 hours).

The metabolism of phospholipids in tumor tissues has been studied (301, 302, 303) using both radioactive phosphorus and elaidic acid as tracers. Relative differences in turnover of various phospholipids were found using these two indicators. With P^{32} it was shown that the rate of lecithin turnover was greater than that of cephalin in rat carcinosarcoma 256 (302), and it was suggested that lecithin might be involved principally in metabolism, and cephalin in structural functions of the cell. Sphingomyelin was found in the whole cells but not in the nuclei of this tumor (304). Since radioactive phosphorus was rapidly incorporated into tissue sphingomyelin (305, 306), the conclusion has been drawn that sphingomyelin plays an important role in phospholipid-phosphorus metabolism.

Boron or lithium salts of dyes which localize in tumor tissues and there capture slow neutrons and locally release ionizing energy or radioactive dyes have been studied (11, 307 to 313).

Abnormalities in body metabolism induced by tumors.—The Memorial Hospital group have recently reviewed their series of papers on metabolic abnormalities in patients with cancer of the gastrointestinal tract (314). The plasma vitamin A levels of gastrointestinal cancer patients was found to be significantly lower than normal. The administration of vitamin A did not restore the plasma level to normal, but the administration of yeast, lipocaic, or inositol did (315, 316). The hepatic vitamin A levels of cancer patients were in the normal range (317), indicating an abnormal distribution of vitamin A between liver and plasma. The liver of gastrointestinal cancer patients showed a high incidence of fatty infiltration (314, 318), but this in itself did not appear to be responsible for the abnormal vitamin A distribution; since fatty livers with low vitamin A content may occur, the lipotropic properties of lipocaic could be accounted for by inositol alone (316). The hypoproteinemia observed in gastrointestinal cancer was not due to dietary deficiency (314, 319), but since the glycine tolerance curve indicated a marked delay in glycine absorption (320), was probably due partially to poor absorption of amino acids. The hypoproteinemia was also partly attributable to a defect in protein synthesis since the incidence of hepatic dysfunction was high (321, 322, 323). The hypoproteinemia, which persists postoperatively for a considerable time, does not appear to be the result of excessive protein breakdown (314,

324, 325). The achlorhydria frequently found in patients with gastric cancer may be due to the presence of a gastric secretory depressant (326). Intravenously administered glycine increased urinary output of creatine and creatinine by normal patients and patients with benign gastrointestinal disorders but did not do so (even with choline also supplied as methyl group source) in the case of patients with gastrointestinal cancer and coexisting hepatic cirrhosis (327).

An abnormal metabolism of ascorbic acid in cancer is suggested by observations that prolonged administration of a large amount of ascorbic acid was necessary to cause tissue saturation (constant urinary output), and after saturation the differences between administered and excreted ascorbic acid were greater in cancer patients than in normals (93, 328, 329). Similar changes in ascorbic acid metabolism have been reported for other diseases.

No consistent change in the glucose tolerance test in clinical cancer cases was found (330), although the disappearance of glucose from the blood stream seemed slower in the group of patients with the most anaplastic tumors. However, a disturbance of carbohydrate metabolism is suggested by the great increase in urine acidity found in gastric carcinoma patients after ingestion of carbohydrate (331, 332). It has likewise been observed that the blood pyruvate rises several-fold soon after rats are implanted with Jensen sarcoma (333, 334). Human leukemic white cells utilized more pyruvate than did normal white cells, but converted less of it to lactate (44), although urinary excretion of thiamin and pyrimidine accelerators was normal (93).

Sterol balance studies in normal and tumor-bearing mice (335) showed no differences between the two groups. However, when the food intake of control rats was restricted to that of litter mates bearing transplanted Walker adenocarcinoma 256, it was found that the tumor-bearing animals synthesized significantly more sterol than the controls (336). An elevation in cholesterol excretion in eight out of thirty-two cancer patients has been reported (337). Δ^5-Androstenetriol-3(β)16,17 has been isolated from urine of a case of adrenocortical carcinoma (338).

The growth of tumors in rats caused a decrease in the proportion of highly unsaturated fatty acids in the subcutaneous fat (339) as well as a decrease in total lipids in the skin and carcass of rats on a fat-free diet (340). The growth of the tumors was not decreased by a diet free of fat for ninety-seven weeks (340).

Aspects of Carcinogenic Mechanism

Chemical, physical, and biological factors.—It would be quite beyond the scope of this chapter to report on or give references to the vast number of papers since 1939 dealing with the structural chemistry and synthesis of carcinogens, with problems relating chemical structure to carcinogenic action, and with strictly biological results of tests of individual, synthetically prepared carcinogens (tests to 1940 reviewed by Hartwell [6]); nor can results be detailed on many types of possible carcinogenic agents, other than the synthetically prepared ones, that have been applied by feeding, injection, painting, implanting, inhaling, or irradiation, e.g., metals—chromium, arsenic, cobalt (341); salts of arsenic (342, 343), selenium (344), zinc (345), copper (346), and thorium (347); carbon tetrachloride (348); urethane (349); triphenylethylene (350, 351); sulfanilamide (352); ergot (353); furfural (354); bile acids (355); chewing tobacco (356); tobacco smoke (357); tars of tobacco (358, 359), wood smoke (360), tea (361), and maté weed (362); other foods (363); fuel oil distillate (364); heated oils (365); oil and water colors (366); croton resin (367, 368); creosote oil (369), cod liver oil (370); necrotic spleen (371); various dusts (372 to 376); toxic gases (377); radon seeds (378).

Considerable progress has been made in correlating chemical structure and carcinogenic action (6, 379 to 393). It seems likely that the most useful relationships and principles will be obtained from a study of closely related series of analogous compounds. In the past, in seeking for the dominant factors in carcinogenic action, too much attention has probably been paid to structural differences as compared to other chemical, physical, or physiological effects, e.g., fluorescence (394, 395, 396), ultraviolet absorption (397), optical rotation (398), ion or salt formation (399), conjugation with sulfhydryl compounds (400) or proteins (401, 402, 403), mechanical effects (404), solvents and vehicles (405 to 409), limiting doses (408, 410), local and constitutional effects (411), cauterization (412), and mutations (413).

Excellent and indispensable as the work on synthesis and testing of new polycyclic hydrocarbons has been, it is a great weakness of work in this field that, as compared to structural considerations, relatively little attention has been paid to the many other chemical, physical, and metabolic properties of these compounds (see, however,

13), in an effort to establish broader principles of chemical carcinogenic action than could ever be covered by empirical structural rules alone. The limited structural outlook and indicated errors of omission in this field have induced a certain sense of stasis and pessimism that does not seem at all warranted so far as future research on chemical carcinogenesis is concerned. The time is obviously ripe to effectively correlate carcinogenesis of already tested compounds with their many important properties besides structure.

One of the most promising lines of investigation dealing with chemical principles of carcinogenic action going beyond structural considerations is that carried out by Kensler and co-workers (414, 415, 416), Potter (417), and Cohen *et al.* (125) on the effect of split products of carcinogenic azo dyes on the activities of enzyme systems. The known or postulated split products of *p*-dimethylaminoazobenzene inhibit the action of a yeast phosphopyridine nucleotide system (415), transaminase (125), carboxylase (416), succinoxidase (417), urease (417), and other enzymes (414). It is quite possible that the inhibition is due to the combination of the split products with sulfhydryl groups in enzyme proteins of the type of triosephosphate dehydrogenase. The inhibition of the diphosphonucleotide system by a series of (nonacetylated) aromatic amines related to *p*-phenylenediamine was closely correlated with the stability of their semiquinone (free radical), intermediary oxidation products, and this in turn with the carcinogenic potency of the respective parent azo compounds! Kuhn & Beinert (418) believe that fully oxidized quinone forms must also be considered in regard to enzyme toxicities, but give no data correlating with carcinogenicity; moreover, Kensler *et al.* (415) found that cysteine overcame inhibition by quinone but not by N, N-dimethyl-*p*-phenylenediamine.

The oxidation of lipids or aldehydes, including catalysis thereof by ascorbic acid and similar biological agents, is frequently inhibited by a wide variety of carcinogens (419 to 423) which, it would appear, often undergo change themselves, thereby. The different effects of various oils used as solvent vehicles (405, 406, 424) or in diets (425) in applying carcinogens on the degree of carcinogenesic result obtained may well be connected with oxidation reactions involved.

The group at the Barnard Hospital have initiated a systematic study to determine and integrate alterations in histological, physical, and biochemical properties of mouse skin treated with methylcholanthrene with a view to building a comprehensive picture of the

modifications that lead to cancer in the epidermal cells (426, 427, 428). Studies on nuclear viscosity (429), cellular and nuclear size (430), mitotic frequency (431), lipoid-protein ratio (432), thymonucleic acid (433), sodium and calcium (434), magnesium and potassium (435), and iron and ascorbic acid (436) have indicated that changes in skin biochemistry appear very shortly after the application of methylcholanthrene. Calcium and iron contents dropped to about one half of normal (on a nucleoprotein phosphorus basis) within ten days, whereas the other chemical constituents showed only minor variations during the experimental period leading up to the formation of gross tumors. Similar experiments with respect to benzpyrene and mineral metabolism and other effects in mice have been described by Aivasian (437, 438).

Perhaps the most significant, single, recent contribution to carcinogenesis is represented by the work of Earle and collaborators (262, 439 to 442) who have obtained the regular *in vitro* production of highly malignant sarcomas in mice upon injection of various strains of normal mouse fibroblast tissue cultures, some, but not all, of which had been treated with 20-methylcholanthrene. Although a number of previous investigators, studying the action of chemical carcinogens on normal tissue cultures, had observed certain cell changes (443), the present publications are the first record of a series of changes which resulted in cells so altered that on injection into animals they promptly and consistently gave rise to malignant tumors that were grossly and histologically similar to sarcomas previously reported as having arisen spontaneously or as having been induced with carcinogens in mice. The neoplasms induced *in vitro* had the ability to invade surrounding structures, to produce metastases in instances, and, in advanced cases, cause death of the animal; and as already noted, the metabolism of the tumors has been found (40) to be characteristic of highly malignant tumors generally. The methylcholanthrene definitely produced progressive changes in the tissue culture cells that persisted even after the methylcholanthrene was removed, but whether the induction of malignancy resulted from minute amounts of residual (not grossly accidental) contamination of the control cultures with methylcholanthrene, or required no methylcholanthrene at all is still under investigation. Particularly needed is information on how long a latent period is required to obtain tumors from the tissue culture fibroblasts. The broad significance of the present research lies in its having shown that neither the host organism nor material (in-

cluding hormones, and possibly viruses) derived from the host organism is necessary for the development or maintenance of malignancy in tissue cells. Furthermore, the malignant state, as evidenced by tumor production, was apparently acquired by the great bulk of the cells experiencing the same changes rather uniformly, instead of uniquely and randomly as in mutation. The value of this research lies fully as much in the issues it raises as in those it meets (444).

Of a similar degree of interest, and requiring independent confirmation, is the claim of Taylor *et al.* (245, 445, 446, 447) that transplanted dba mouse mammary tumors cultivated in the yolk sac of developing chicken eggs liberated a virus-like principle that produced tumors in dba mice when injected either as an N-size Berkefeld filtrate (445) or as a lyophilizate (446). The rapid appearance of tumors at the site of injection seemingly distinguishes this material from the slowly acting chemical carcinogens and possibly from the latent virus of mouse mammary cancer ("milk factor," see below). Nonidentity with the milk factor latent virus would be indicated if and when other types of tumors are demonstrated to yield similar cell-free propagating agents or viruses.

Latent virus of mammary tumors.—The tendency of certain strains of mice to have a high or a low incidence of spontaneous mammary tumors, once thought to be chiefly an expression of the genetic constitution of the strains, has been clearly shown to depend also upon a factor ingested with the milk of the mother, a so-called extrachromosomal factor (see reviews, 22, 448 to 452). This cell-free sedimentable agent that increases the incidence of mammary tumors in mice, designated as the mammary tumor inciter, has been reported not only in milk but in several tissues and in blood of high cancer strain mice (451, 453 to 459). The mammary tumor agent has been reported to be filtrable through a Seitz filter (455) and to withstand lyophilization (454). It was completely sedimented in the ultracentrifuge at 110,000 times gravity for one hour (459), but at 60,000 times gravity for one hour an appreciable portion remained in the supernatant liquid (458), and Andervont & Bryan (unpublished) have found that pasteurization of mouse milk (60° C. for 30 minutes) destroyed the activity. Andervont (460) has reported that strain C3H milk influence was transferred through at least two generations of resistant, strain C, albino females, and his further unpublished work has now indicated that this influence can be passed through

at least nine generations of this strain, indicating that the agent is self-propagating. Fekete & Little (461) reported that C57 black mice, when initially exposed to both intra-uterine and milk agents of the dba strain, developed tumors through at least four generations of mother to daughter nursing. Studies of the chemical nature, physical properties, and biological behavior of the mammary tumor agent are compatible with the view that the agent is a latent virus (454, 458, 459, 462).

A somewhat similar foster nursing effect has been reported for certain strains of transplantable leukemia in mice (463, 464), as has a less related maternal influence in spontaneous leukemia in mice (465, 466, 467).

Endogenous carcinogens.—The possibility that cancer may be induced by carcinogenic substances occurring naturally in tissues and body fluids has been studied experimentally by several investigators and the subject has recently been reviewed by Steiner (468). Lipid extracts capable of producing cancer in mice at the site of injection have been prepared from human livers, particularly those of patients dying with malignant disease (469 to 478). Lipids extracted from various cancers have also been found to be carcinogenic in mice (479 to 483) and rats (478). The presence of carcinogenic substances in the lipids of urine from patients with or without cancer has also been claimed (484), although other attempts to extract such a carcinogenic substance from urine were not successful (485, 486). Cancers have also been produced in mice by the injection of the nonsaponifiable lipids of human bile and gall bladders (473) and of whole ox bile (487). Little is known of the nature of the compounds responsible for the carcinogenic action of the lipid extracts. A suggestion (488, 489) that the fluorescence spectra of liver extracts showed bands resembling those of the carcinogen methylcholanthrene has been severely criticized (490).

The carcinogenic factor in liver has appeared mainly in the nonsaponifiable lipids (472 to 475, 480), whereas the active factor in several tumor lipid extracts appeared in the whole extract but not in the nonsaponifiable fraction (483), suggesting that different agents may be responsible. Injection of material isolated from inflammatory exudates into rabbits' ears led to proliferations resembling neoplasms (491). Substances causing myeloid proliferations when injected into animals have been obtained from the urine and feces of leukemia patients (492, 493, 494), and substances causing sarcomas

in rats have been obtained by benzene extraction of coli bacteria (495).

It is possible that tissue extracts may contain carcinogenic compounds derived from sex hormones, bile acids, cholesterol, and other sterols. It is yet too early to draw many conclusions from the demonstration of the presence of carcinogenic substances in tissue extracts and body fluids; whether or not such substances play a definite role in clinical cancer must still be determined by future research.

Metabolism of carcinogens.—Studies on the metabolism of carcinogenic compounds have yielded information on how certain of these compounds are detoxified and excreted from the body. Following the observation of Hashimoto (496) that the carcinogen *o*-aminoazotoluene was split at the azo linkage in the rat, Stevenson, Dobriner & Rhoads (497) concluded that *p*-dimethylaminoazobenzene was also split at the azo linkage since aminophenol and *p*-phenylenediamine in both the free and acetylated forms were found in the urine of *p*-dimethylaminoazobenzene-fed rats. No *p*-dimethyl-*p*-phenylenediamine was found in the urine, but it was assumed that this compound was the precursor of the excreted *p*-phenylenediamine. However, the demethylation might conceivably occur before the splitting of the azo linkage. That the methyl groups of *p*-dimethylaminoazobenzene might be available for the body was indicated by the discovery that *p*-dimethylaminoazobenzene ingestion prevented the hemorrhagic kidney degeneration of rats fed on a low-methionine, low-choline diet (498). The interesting observation (499, 500, 501) that the cystine-methionine level of rat-diets markedly influences the growth inhibiting effect of various carcinogenic and noncarcinogenic hydrocarbons suggests that the formation of mercaptans is involved in the detoxification of these substances. However, acceptance of this suggestion must await the isolation of such compounds from the urine and feces of hydrocarbon-fed rats. It is interesting in this connection that Fieser and co-workers synthesized the mercapturic acid derivatives of benzpyrene (502) and found them to be noncarcinogenic.

A fluorescent substance, BPX, was discovered in the bile, feces, and urine of animals injected with 3,4-benzpyrene (404, 503 to 507) and was considered to be a monohydroxy derivative of benzpyrene (508). This substance as well as a red quinone derivative of benzpyrene was demonstrated in the feces of mice injected intraperitoneally with 3,4-benzpyrene (509, 510). The carcinogen apparently passed to the liver, where it was largely metabolized and excreted into the

digestive tract via the bile. The monohydroxy benzpyrene derivative was isolated, and it was shown that the hydroxyl group was in the 8 position and that the red quinone was 3,4-benzpyrene-5,8-quinone (511, 512), and that BPX was 8-hydroxy-3,4-benzpyrene.

It appears (511) that the greater part of the benzpyrene lost from the body appears in the excreta in the form of 8-hydroxy-3,4-benzpyrene or 3,4-benzpyrene-5,8-quinone, neither of which has been found to be very carcinogenic. It was observed that 3,4-benzpyrene injected or painted on the surface of mouse skin was transformed into a blue-fluorescent, alkali-soluble compound that persisted long after the original benzpyrene had disappeared (513, 514, 515), and was different from BPX, and it was suggested as an intermediate stage in the local metabolism of 3,4-benzpyrene (516). The different postulated intermediates were partially separated by adsorption on aluminum oxide but their nature has not yet been determined.

The metabolism of 1,2,5,6-dibenzanthracene has also been shown to involve the formation of hydroxy derivatives that are excreted principally in the feces. Dihydroxydibenzanthracene has been isolated from excreta of rats, mice, and rabbits injected with 1,2,5,6-dibenzanthracene (517 to 520). The derivative isolated from rat and mouse excreta (520) was identical with 4′,8′-dihydroxy-1,2,5,6-dibenzanthracene synthesized by Cason & Fieser (521). The metabolite isolated from rabbit excreta probably differed from the above derivative by having the hydroxyl groups in different positions (519, 520). The rabbit dihydroxydibenzanthracene is not identical with 3,7-dihydroxy-1,2,5,6-dibenzanthracene synthesized by Cason & Fieser (522), and it seems likely that the hydroxyl groups are in the 3- and 8- positions.

Jones (523) was able to account spectroscopically only for about 6 per cent of the 1,2,5,6-dibenzanthracene injected into rats as unchanged carcinogen and its dihydroxy derivative in the urine and feces, an observation that might suggest that dibenzanthracene may be excreted as other derivatives. However, further studies (524) indicated that the low recoveries might have been due to the retention of dibenzanthracene in vesicles near the site of injection. Intraperitoneally administered 1,2-benzanthracene is excreted in the feces as a phenolic derivative that is probably 4′-hydroxy-1,2-benzanthracene (525).

Chang & Young (526, 527, 528) have reported that naphthalene-, phenanthrene-, and anthracene-fed rats excreted in the urine an ether-

insoluble material that liberated the original hydrocarbon on treatment with acid. 3,4-Benzpyrene, 1,2,5,6-dibenzanthracene, and methylcholanthrene did not form compounds with these properties.

Metabolic derivatives of other carcinogenic hydrocarbons have not been isolated, but the detection of alkali-soluble fluorescent products in the bile of fowls injected with several hydrocarbons (529) suggested that similar metabolic changes are involved which also give rise to hydroxy derivatives that are noncarcinogenic and readily excreted.

Lorenz & Shimkin (530) have investigated the rate of elimination of intravenously injected methylcholanthrene from mice of two strains which differ markedly in their susceptibility to methylcholanthrene induced lung tumors (strains A and C57 black). It was observed that the carcinogen disappeared from the lungs and whole carcasses at the same rate. The difference in the cancer susceptibility, then, could not be accounted for on the basis of different rates of methylcholanthrene elimination.

Unsaturated fatty acids in diets have been reported to destroy *p*-dimethylaminoazobenzene, but rice contains a stabilizer and hence inclusion of rice in the diet renders it procarcinogenic (531).

Chemotherapy

Bacterial filtrates.—Of a number of substances which elicit hemorrhage in primary and transplanted tumors in mice, the most potent as a class have been obtained from filtrates of a variety of bacterial cultures (532 to 545). Such substances also induce the Shwartzman phenomenon in rabbit skin, and it is now strongly indicated that the agents in the preparations responsible for both effects are identical or closely related (546).

Concentrates of very high tumor-hemorrhage-producing potency have been prepared by Shear and associates from filtrates of *Serratia marcescens* grown in simple synthetic medium (535, 537). As little as 0.1 μg. of these preparations caused hemorrhage in mouse sarcoma 37 in 50 per cent of the tumors. The material was found to be a polysaccharide containing about 2 per cent nitrogen that was largely accounted for as hexosamine nitrogen and by the nitrogenous component of a phospholipid that was firmly bound to the polysaccharide (537). This is contrary to the suggestion that the hemorrhage-producing material is probably identical with a polypeptide component

of certain complete "endotoxin" nonprotein 0 antigens (539, 543). The preparations were found to be homogeneous in ultracentrifugal and electrophoretic analyses (538). The particles were markedly asymmetric in shape, the ratio of the long to short axes being in the range 16 to 21 to one. The molecular weight of the material lay in the range of 1 to 10 million, although evidence of aggregation made this a probable maximum. It has been reported that sulfanilamide raised the minimal hemorrhage-producing doses in mouse sarcoma 180, and also the minimum lethal dose of fractions obtained from cultures of *Salmonella typhyimurium* (541, 543). The remarkable property of these bacterial toxins in bringing about hemorrhage, necrosis, and sometimes regression of experimental tumors at doses only 1/100 to 1/1000 of the lethal dose does encourage the hope that such agents may eventually be of some chemotherapeutic value. Little is known of the mechanism by which the bacterial toxins produce hemorrhage in tumors, or of the reason for the selective action on tumors, but it seems likely that differences in the fragility of the vascular bed may account for the increased sensitivity of the tumors. An earlier suggestion that ascorbic acid exerted a protective effect against tumor hemorrhage (547) has not been confirmed using more highly purified preparations (537). The hemorrhages do not appear to be due to the thrombocytopenia induced by the bacterial filtrates since reduction of the platelet count by anti-mouse platelet serum did not produce hemorrhage in sarcoma 37 (548). Tumors implanted in mice previously immunized with *Shigella paradysenteriae* Flexner were found, in contrast to those implanted in nonimmunized mice, to be highly resistant to the tumor-hemorrhage factor of the Flexner organism (542).

Production of hemorrhage in tumors is not restricted to bacterial filtrates since colchicine, in amounts close to the lethal dose, also gives similar effects (532, 549, 550, 551). Hemorrhage in tumors has also been elicited by tissue extracts (552) and by moccasin venom (548). Likewise, hemorrhage and necrosis in sarcoma 37 were observed when tumor bearing mice were injected with very large amounts of histamine (10 mg.) or peptone (40 mg.); and tumor hemorrhage also occurred in tumors of mice bearing sarcoma 37 when they were brought into anaphylactic shock by injection with horse serum after previously being sensitized to horse serum (553). That the hemorrhage produced by bacterial filtrates is likewise an anaphylactic response is made somewhat unlikely by the observation that shock

is not evident in mice given amounts of bacterial filtrate necessary to elicit tumor hemorrhage.

Tissue extracts and amino acids.—A number of investigators have recently reported that the injection of tissue extracts may bring about a decrease in the growth rate of experimental tumors and frequently also causes their regression. Lewisohn and collaborators have extended their earlier observations and have shown that extracts of spleen, yeast, and grains may cause reduced growth and actual regression of a certain percentage of mouse sarcoma 180, mouse adenocarcinoma 2136, and spontaneous mouse mammary carcinomas (552, 554 to 561), with somewhat similar results with inositol in large doses (562). The factor in yeast was found to be water soluble and would diffuse through a cellophane membrane, indicating that it was not a protein (554). It was apparently not inactivated by nitrous acid. It seemed to be precipitated by high concentrations of alcohol and by phosphotungstic acid, but not by lead acetate or silver nitrate. The active factor was destroyed by 0.5 *N* hydrochloric acid in three hours at room temperature.

Boyland (563, 564) has reported that orally administered protein-free extracts of muscle inhibited the growth of transplanted sarcomas and spontaneous mammary carcinomas in mice. A number of organic bases were tested, and it was found that ethanolamine and cadaverine hydrochloride were the most effective inhibitors of growth of tumors. Dobrovolskaïa-Zavadskaïa & Zephiroff (565 to 568) report studies on tumor inhibition by extracts of liver, adrenal gland, and pineal gland, and by urine, as have also others with urine (569, 570, 571), and with ether-extracted wheat germ oil (572).

The intravenous injection of a number of amino acids, particularly those essential to the nutrition of the rat, has been claimed to inhibit the growth, and to cause some regressions, of the Emge sarcoma in rats (573, 574, 575). On the other hand, it has been reported that proline (one of the alleged protective amino acids) had a slight stimulating action on the incidence of spontaneous mammary tumors in mice (576, 577), whereas methionine sulfoxide had no effect upon the incidence or growth of spontaneous mammary tumors in mice (578).

Heptaldehyde.—Further confirmation that administration of heptaldehyde, alone or in combination with sodium bisulfite, may cause liquefaction and regression of certain spontaneous and transplanted tumors in mice has been reported (564, 579 to 582), although all

tumors do not appear to be affected (583, 584). The administration of heptaldehyde is accompanied by debatable renal damage (582, 584). Heptaldehyde also brought about the resorption of embryos in pregnant mice and rats (583, 585). Boyland (581) postulated an oxidation of heptaldehyde *in vivo* by way of pimelic, glutaric, and malonic acids. In support of this suggestion it was found that these acids had marked inhibitory effects on the growth of spontaneous and transplanted tumors. Boyland (563, 564, 581) has observed that citral, another aldehyde, was considerably more effective than heptaldehyde in inhibiting the growth of spontaneous mammary tumors in mice. Of various possible metabolic derivatives studied, none showed significant tumor inhibiting activity (581).

Other agents.—Earlier claims that an organic peroxide (dihydroxymethyl peroxide) could inhibit the growth of tumors have been refuted by Belkin (586, 587, 588) using a great number of mice and several types of tumors. The plant hormones, 3-indolebutyric acid and 3-indolepropionic acid, were found to destroy the ability of tumor cells to be transplanted into new hosts (589, 590), and have also been reported to have some inhibiting effect on the rate of tumor growth when injected into tumor-bearing animals (590, 591, 592). Inhibition of tumor growth has further been reported for carcinogenic and related noncarcinogenic compounds (593 to 597), colchicine (595, 598, 599), bacteriophage (600), parenterally administered antigenic proteins (601), glycogen (602), nitrosophenols and aromatic diamines (564), ammonium, lithium, and magnesium salts (603, 604), monochloracetone and various acid chlorides (605, 606), and heavy metals (607). Extensive, systematic, chemotherapeutic studies of effects of chemicals, x-rays, and underfeeding on transmitted mouse leukemias have provided interesting results, especially in regard to benzene and strain differences (597, 608, 609). The use of radiophosphate for therapeutic treatment of leukemia and allied disorders, already considered, has been reviewed in detail (294). The role of hormones and dietary constituents, regarded as therapeutic agents in the broadest sense, is very important but cannot be treated here.

Avidin.—The suggestion has been advanced that cancer might in instances be controlled by the deprivation of biotin brought about through the oral administration of avidin (610) either as a concentrate or as egg white. This suggestion was based primarily on the assumption that cancer might have an abnormally high biotin requirement and might thus be selectively inhibited by an induced biotin deficiency.

The suggestion was also in line with the observation that the addition of biotin to diets that protected against the formation of hepatomas in rats fed *p*-dimethylaminoazobenzene increased the incidence of liver tumors very significantly (611, 612), and also with the more recent observation that a percentage of regressing transplanted hepatomas were induced to resume growth by biotin supplementation (4).

Favorable changes in a small number of tested cancer patients given large amounts of egg white in their diet have been claimed (613, 614). However, other cancer patients were apparently not benefited by the oral administration of large amounts of avidin and egg white (615). In these clinical studies, however, frank biotin deficiency symptoms were not obtained. In experimental studies with mice and rats (and tumor tissue cultures *in vitro*), it was reported that neither the incidence, growth rate, nor regression of spontaneous mammary carcinomas, transplanted sarcomas, nor Flexner-Jobling carcinomas was altered in animals showing biotin deficiency symptoms due to avidin or egg white feeding (31, 616). Curiously enough, however, certain of the data (616, table 1) indicate a quite definite decrease in mammary tumor incidence (31 to 14 per cent) and increase in spontaneous regression (0 to 8 per cent) upon avidin–egg white treatment. Of some importance here may be the very recent observation of Schade & Caroline (617) that egg white is capable of inducing iron deficiency in lower (and hence possibly higher) organisms by combination with iron; avidin, which combines so effectively with biotin, is not the component in egg white responsible for the efficient combination with iron. The work of Myer (618) and Laurence (619) indicating a close relationship (though scarcely identity) between avidin (or avidin-biotin) and lysozymes, and possibly hyaluronidase or spreading factor (123), likewise suggests much experimentation.

The hope of avidin therapy for cancer was based partly on the assumption that tumors have an abnormally high biotin content or requirement, but this does not seem to be a general phenomenon (24, 31, 39, 40, 41). A real difficulty with avidin therapy in patients is evidently that it cannot or has not produced sufficiently great biotin deficiencies to be of critical interest. A much more hopeful approach is indicated in the use of antivitamers of biotin (viz., compounds that act to overcome biotin activity), as outlined elsewhere (4). Burk & Winzler (39) have suggested that miotin, the main avidin-uncombinable, autoclaving-labile, unidentified material in urine and tissues that possesses biotin vitamer activity for yeast (viz., acts to overcome

biotin deficiency therein), may be an antivitamer of biotin with respect to higher animals, and might be able, if isolated and applied in sufficient quantity, to competitively eliminate biotin in tumors or normal tissues far more effectively than does avidin. Likewise, the biotin vitamer of known chemical structure, desthiobiotin, which has been shown to act as a biotin vitamer for yeast but as a biotin antivitamer for *L. casei* (620, 621), might also act antivitamerically with respect to biotin in tumor growth; and the same may be said of still other untested but possible antivitamers of biotin, or still more broadly, of antivitamers of various other vitamins in general.

LITERATURE CITED

1. BOYLAND, E., *Ann. Rev. Biochem.*, **3**, 400–9 (1934)
2. HOLMES, B. E., *Ann. Rev. Biochem.*, **4**, 469–78 (1935)
3. DODDS, E. C., AND DICKENS, F., *Ann. Rev. Biochem.*, **9**, 423–58 (1940)
4. BURK, D., AND WINZLER, R. J., *Vitamins and Hormones*, Vol. II, pp. 305–52. (Academic Press, Inc., New York, 1944)
5. DODDS, E. C., *Vitamins and Hormones*, Vol. II, pp. 353–59. (Academic Press, Inc., New York, 1944)
6. HARTWELL, J. L., *Survey of Compounds Which Have Been Tested for Carcinogenic Activity*, 371 pp. (Federal Security Agency, United States Public Health Service, National Cancer Institute, 1941)
7. STERN, K., AND WILLHEIM, R., *The Biochemistry of Malignant Tumors*, 951 pp. (Reference Press, Brooklyn, New York, 1943)
8. VON EULER, H., AND SKARZYNSKI, B., *Biochemie der Tumoren*, 260 pp. (Ferdinand Enke Verlag, Stuttgart, 1941)
9. MENIKOV, A. V. (Editor), *General and Special Oncology*, Vol. **I**, 615 pp. ("Medgiz," Narkomzdrav, Moscow-Leningrad, U.S.S.R., 1940); *Cancer Research*, **4**, 71 (1944)
10. BOGOMOLETZ, A. A., KAVETSKY, R. E., AND MELNIKOV, A. V. (Editors), *The Works of the First Convention of Oncologists of the Ukrainian Soviet Socialist Republic*, 547 pp. ("Medgiz," Narkomzdrav, Moscow-Leningrad, U.S.S.R., 1940); *Cancer Research*, **4**, 71–72 (1944)
11. GREENSTEIN, J. P., *J. Natl. Cancer Inst.*, **3**, 419–47 (1943)
12. LUSTIG, B., AND WACHTEL, H., *Protoplasma*, **32**, 556–97 (1939)
13. DITTMAR, C., *Protoplasma*, **32**, 598–629 (1939)
14. KÖHLER, K., *Handb. Enzymolog.* (*Leipzig*), **2**, 1160–214 (1940)
15. VON EULER, H., *Angew. Chem.*, **53**, 352–55 (1940)
16. BUTENANDT, A., *Angew. Chem.*, **53**, 345–52 (1940)
17. CASPERSSON, T., AND SANTESSON, L., *Studies on Protein Metabolism of the Cells of Epithelial Tumors*, 105 pp. (P. A. Norstedt and Söner, Stockholm, 1942)
18. RONDONI, P., *Soc. ital. progresso sci. Scienza Tecnica*, **5**, 721–37 (1941)
19. VOEGTLIN, C., *J. Natl. Cancer Inst.*, **2**, 309–15 (1942)
20. MURPHY, J. B., *J. Am. Med. Assoc.*, **120**, 107–11 (1942)
21. COWDRY, E. V., *Arch. Path.*, **30**, 1245–74 (1940)
22. ROUS, P., *J. Am. Med. Assoc.*, **122**, 573–81 (1943)
23. STEIGERWALDT, F., *Monatschr. Krebsbekämpf.*, **11**, 1–13, 21–31 (1943)
24. POLLACK, M. A., TAYLOR, A., WOODS, A., THOMPSON, R. C., AND WILLIAMS, R. J., *Cancer Research*, **2**, 748–51 (1942)
25. POLLACK, M. A., TAYLOR, A., TAYLOR, J., AND WILLIAMS, R. J., *Cancer Research*, **2**, 739–43 (1942)
26. POLLACK, M. A., TAYLOR, A., AND WILLIAMS, R. J., *Univ. Texas Pub.*, **4237**, 56–71 (1942)
27. TAYLOR, A., POLLACK, M. A., HOFER, M. J., AND WILLIAMS, R. J., *Cancer Research*, **2**, 744–47 (1942)
28. TAYLOR, A., POLLACK, M. A., HOFER, M. J., AND WILLIAMS, R. J., *Cancer Research*, **2**, 752–54 (1942)

29. Taylor, A., Pollack, M. A., and Williams, R. J., *Univ. Texas Pub.*, **4237**, 41–55 (1942)
30. Taylor, A., Pollack, M. A., and Williams, R. J., *Science*, **96**, 322–23 (1942)
31. West, P. M., and Woglom, W. H., *Cancer Research*, **2**, 324–31 (1942)
32. West, P. M., and Woglom, W. H., *Science*, **93**, 525–27 (1941)
33. Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, **91**, 623 (1940)
34. Kahler, H., and Davis, E. F., *Proc. Soc. Exptl. Biol. Med.*, **44**, 604–6 (1940)
35. Robertson, W. v.B., and Kahler, H., *J. Natl. Cancer Inst.*, **2**, 595–600 (1942)
36. Morris, H. P., and Robertson, W. v.B., *J. Natl. Cancer Inst.*, **3**, 479–89 (1943)
37. Leeman, H., *Klin. Wochschr.*, **21**, 60–62 (1942)
38. Burk, D., and Winzler, R. J., *Science*, **97**, 57–60 (1943)
39. Burk, D., and Winzler, R. J., *Gibson Island Research Conf.* (July 30, 1943)
40. Burk, D., Earle, W. R., and Winzler, R. J., *J. Natl. Cancer Inst.*, **4**, 363–72 (1943)
41. Kidd, J. G., Winzler, R. J., and Burk, D., *Cancer Research*, **4** (In press)
42. Vilter, S. P., Koch, M. B., and Spies, T. D., *J. Lab. Clin. Med.*, **26**, 31–44 (1940)
43. Abels, J. C., Gorham, A. T., Craver, L. F., and Rhoads, C. P., *J. Clin. Investigation*, **21**, 177–89 (1942)
44. Abels, J. C., Jones, F. L., Craver, L. F., and Rhoads, C. P., *Cancer Research*, **4**, 149–52 (1944)
45. Robertson, W. v.B., *J. Natl. Cancer Inst.*, **4**, 321–28 (1943)
46. Goldstein, B. J., Volkenson, D. V., and Kacherova, S. A., *Biochem. J. U.S.S.R.*, **17**, 216–17 (1941)
47. Butler, A. M., and Cushman, M., *J. Clin. Investigation*, **19**, 459–67 (1940)
48. Kishi, S., and Nakahara, W., *Gann*, **34**, 252-55 (1940)
49. Abels, J. C., Gorham, A. T., Eberlin, S. L., Halter, R., and Rhoads, C. P., *J. Exptl. Med.*, **76**, 143–61 (1942)
50. Baumann, C. A., Foster, E. G., and Lavik, P. S., *J. Nutrition*, **21**, 431–44 (1941)
51. Carruthers, C., *Cancer Research*, **2**, 168–74 (1942)
52. Goerner, A., and Goerner, M. M., *J. Biol. Chem.*, **128**, 559–65 (1939)
53. Goerner, A., and Goerner, M. M., *Am. J. Cancer*, **37**, 518–20 (1939)
54. Goerner, A., and Goerner, M. M., *J. Nutrition*, **18**, 441–46 (1939)
55. Goerner, A., and Goerner, M. M., *Cancer Research*, **3**, 833–38 (1943)
56. Marshak, A., *J. Gen. Physiol.*, **25**, 275–91 (1941)
57. Goerner, A., and Goerner, M. M., *J. Biol. Chem.*, **123**, 57–59 (1938)
58. Goerner, A., *J. Biol. Chem.*, **122**, 529–38 (1938)
59. Marron, T. U., *Proc. Soc. Exptl. Biol. Med.*, **48**, 219–21 (1941)
60. Wagner-Hering, E., *Hippokrates*, **13**, 344–46 (1942)
61. Popper, H., and Ragins, A. B., *Arch. Path.*, **32**, 258–71 (1941)
62. Seeger, P. G., *Arch. exptl. Zellforsch. Gewebezücht.*, **24**, 59–71 (1940)

63. von Euler, B., and von Euler, H., *Z. physiol. Chem.*, **264**, 141–45 (1940)
64. von Euler, B., and von Euler, H., *Z. physiol. Chem.*, **265**, 147–51 (1940)
65. Vincent, D., Daum, S., and Bouchet, M., *Trav. membres, soc. chim. biol.*, **23**, 1363–68 (1941)
66. Greenstein, J. P., *J. Natl. Cancer Inst.*, **2**, 511–24 (1942)
67. Greenstein, J. P., Jenrette, W. V., and White, J., *J. Natl. Cancer Inst.*, **2**, 17–22 (1941)
68. Greenstein, J. P., and Jenrette, W. V., *J. Natl. Cancer Inst.*, **1**, 845–63 (1941)
69. Greenstein, J. P., and Stewart, H. L., *J. Natl. Cancer Inst.*, **2**, 631–33 (1942)
70. Greenstein, J. P., Edwards, J. E., Andervont, H. B., and White, J., *J. Natl. Cancer Inst.*, **3**, 7–17 (1942)
71. Greenstein, J. P., Jenrette, W. V., Mider, G. B., and White, J., *J. Natl. Cancer Inst.*, **1**, 687–706 (1941)
72. Greenstein, J. P., Jenrette, W. V., Mider, G. B., and Andervont, H. B., *J. Natl. Cancer Inst.*, **2**, 293–99 (1941)
73. Greenstein, J. P., *J. Natl. Cancer Inst.*, **2**, 357–59 (1942)
74. Greenstein, J. P., *J. Natl. Cancer Inst.*, **4**, 55–61 (1943)
75. Greenstein, J. P., and Thompson, J. W., *J. Natl. Cancer Inst.*, **4**, 271–74 (1943)
76. Greenstein, J. P., and Thompson, J. W., *J. Natl. Cancer Inst.*, **4**, 275–81 (1943)
77. Greenstein, J. P., Jenrette, W. V., and White, J., *J. Natl. Cancer Inst.*, **2**, 283–91 (1941)
78. Greenstein, J. P., and Andervont, H. B., *J. Natl. Cancer Inst.*, **2**, 345–55 (1942)
79. Greenstein, J. P., *J. Natl. Cancer Inst.*, **2**, 525–30 (1942)
80. Greenstein, J. P., Andervont, H. B., and Thompson, J. W., *J. Natl. Cancer Inst.*, **2**, 589–94 (1942)
81. Greenstein, J. P., and Andervont, H. B., *J. Natl. Cancer Inst.*, **4**, 283–84 (1943)
82. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3**, 397–404 (1943)
83. McEwen, H. D., and Haven, F. L., *Cancer Research*, **1**, 148–50 (1941)
84. Greenstein, J. P., and Thompson, J. W., *J. Natl. Cancer Inst.*, **3**, 405–8 (1943)
85. Shack, J., *J. Natl. Cancer Inst.*, **3**, 389–96 (1943)
86. Maver, M. E., Johnson, J. M., and Thompson, J. W., *J. Natl. Cancer Inst.*, **1**, 675–86 (1941)
87. Westphal, U., *Z. physiol. Chem.*, **276**, 191–204 (1942)
88. Westphal, U., *Z. physiol. Chem.*, **278**, 213–21 (1943)
89. Westphal, U., *Z. physiol. Chem.*, **278**, 222–29 (1943)
90. Westphal, U., and Lang, K., *Z. physiol. Chem.*, **276**, 205–13 (1942)
91. Westphal, U., *Naturwissenschaften*, **30**, 120–21 (1942)
92. Westphal, U., *Naturwissenschaften*, **31**, 117–18 (1943)
93. Strong, L. C., and Francis, L. D., *Am. J. Cancer*, **38**, 399–403 (1940)
94. Lan, T. H., *Cancer Research*, **4**, 37–41 (1944)

95. Lan, T. H., *Cancer Research*, **4**, 42–44 (1944)
96. Figge, F. H. J., and Strong, L. C., *Cancer Research*, **1**, 779–84 (1941)
97. Khanolkar, V. R., and Chitre, R. G., *Cancer Research*, **2**, 567–70 (1942)
98. Chitre, R. G., and Khanolkar, V. R., *Cancer Research*, **3**, 88–91 (1943)
99. Khanolkar, V. R., and Chitre, R. G., *Cancer Research*, **4**, 128–33 (1944)
100. Figge, F. H. J., Strong, L. C., Strong, L. C., Jr., and Shanbrom, A., *Cancer Research*, **2**, 335–42 (1942)
101. Strong, L. C., and Figge, F. H. J., *Science*, **94**, 331 (1941)
102. Elliott, K. A. C., and Greig, M. E., *Biochem. J.*, **32**, 1407–23 (1938)
103. Junowicz-Kocholaty, R., and Hogness, T. R., *J. Biol. Chem.*, **129**, 569–74 (1939)
104. Stotz, E., *J. Biol. Chem.*, **131**, 555–65 (1939)
105. Schneider, W. C., and Potter, V. R., *Cancer Research*, **3**, 353–57 (1943)
106. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, **150**, 131–41 (1943)
107. Dubois, K. P., and Potter, V. R., *Cancer Research*, **2**, 290–93 (1943)
108. Craig, F. N., Bassett, A. M., and Salter, W. T., *Cancer Research*, **1**, 869–79 (1941)
109. Roskelley, R. C., Mayer, N., Horwitt, B. N., and Salter, W. T., *J. Clin. Investigation*, **22**, 743–51 (1943)
110. Albaum, H. D., and Potter, V. R., *Cancer Research*, **3**, 303–8 (1943)
111. Elliott, K. A. C., *Biochem. J.*, **34**, 1134–41 (1940)
112. Woodard, H. Q., and Kenney, J. M., *Am. J. Roentgenol. Radium Therapy*, **47**, 227–39 (1942)
113. Edwards, J. E., and Dalton, A. J., *J. Natl. Cancer Inst.*, **3**, 19–41 (1942)
114. Edwards, J. E., Dalton, A. J., and Andervont, H. B., *J. Natl. Cancer Inst.*, **2**, 555–63 (1942)
115. Kabat, E. A., and Furth, J., *Am. J. Path.*, **17**, 303–18 (1941)
116. Landow, H., Kabat, E. A., and Newman, W., *Arch. Neurol. Psychiat.*, **48**, 518–30 (1942)
117. Gomori, G., *Proc. Soc. Exptl. Biol. Med.*, **42**, 23–26 (1939)
118. White, J., Dalton, A. J., and Edwards, J. E., *J. Natl. Cancer Inst.*, **2**, 539–54 (1942)
119. Woodard, H. Q., *Cancer Research*, **3**, 159–63 (1943)
120. Weil, L., *J. Biol. Chem.*, **138**, 375–80 (1941)
121. Warburg, O., and Christian, W., *Biochem. Z.*, **314**, 399–408 (1943)
122. Kubowitz, F., and Ott, P., *Naturwissenschaften*, **29**, 590–91 (1941)
123. Pirie, A., *Brit. J. Exptl. Path.*, **23**, 277–84 (1942)
124. Kritzman, M. G., and Konikova, A. S., *Arch. sci. biol. (U.S.S.R.)*, **60**, 81–85 (1940)
125. Cohen, P. P., Hekhuis, G. L., and Sober, E. K., *Cancer Research*, **2**, 405–10 (1942)
126. Cohen, P. P., and Hekhuis, G. L., *Cancer Research*, **1**, 620–26 (1941)
127. Bernheim, F., and Felsovanyi, A. v., *Science*, **91**, 76 (1940)
128. Maver, M. E., Mider, G. B., Johnson, J. M., and Thompson, J. W., *J. Natl. Cancer Inst.*, **2**, 277–82 (1941)
129. Maver, M. E., and Thompson, J. W., *J. Natl. Cancer Inst.*, **3**, 383–87 (1943)

130. Konikova, A. S., *Bull. biol. méd. exptl. U.R.S.S.*, **7**, 332–34 (1939)
131. Utzino, S., and Chen, T., *Gann*, **34**, 215–29 (1940)
132. Maver, M. E., and Barrett, M. K., *J. Natl. Cancer Inst.*, **4**, 65–73 (1943)
133. Waldschmidt-Leitz, E., and Mayer, K., *Z. physiol. Chem.*, **262**, Heft 3–5, IV–VI (1939)
134. Waldschmidt-Leitz, E., Hatschek, R., and Hausmann, R., *Z. physiol. Chem.*, **267**, 79–90 (1940)
135. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **132**, 465–66 (1940)
136. Herken, H., and Erxleben, H., *Z. physiol. Chem.*, **264**, 251–53 (1940)
137. Orekhovich, V. N., *Arch. sci. biol. (U.S.S.R.)*, **60**, 123–26 (1940)
138. Ura, S., *Gann*, **35**, 38–40 (1941)
139. Abderhalden, E., and Caeser, G., *Fermentforschung*, **16**, 299–308 (1940)
140. Abderhalden, E., and Abderhalden, R., *Z. physiol. Chem.*, **265**, 253–65 (1940)
141. Abderhalden, E., and Abderhalden, R., *Z. physiol. Chem.*, **270**, 1–8 (1941)
142. Bayerle, H., *Biochem. Z.*, **303**, 251–59 (1939)
143. Bayerle, H., *Biochem. Z.*, **305**, 27–36 (1940)
144. Bayerle, H., *Monatschr. Krebsbekämpf.*, **9**, 1–11 (1941)
145. Bayerle, H., *Monatschr. Krebsbekämpf.*, **9**, 252–56 (1941)
146. Bayerle, H., *Z. Krebsforsch.*, **52**, 341–48 (1942)
147. Bayerle, H., and Podloucky, F. H., *Biochem. Z.*, **304**, 259–65 (1940)
148. Bayerle, H., and Borger, G., *Biochem. Z.*, **307**, 159–69 (1941)
149. Bayerle, H., and Podloucky, F. H., *Z. physiol. Chem.*, **264**, 189–95 (1940)
150. Skarzynski, B., *Arkiv Kemi, Mineral. Geol.*, **13B**, No. 13, 7 pp. (1939)
151. Skarzynski, B., and von Euler, H., *Arkiv Kemi, Mineral. Geol.*, **13B**, No. 17, 8 pp. (1939)
152. Skarzynski, B., and von Euler, H., *Arkiv Kemi, Mineral. Geol.*, **14B**, No. 11, 4 pp. (1940)
153. von Euler, H., Analström, L., Skarzynski, B., and Högberg, B., *Z. Krebsforsch.*, **50**, 552–64 (1940)
154. von Euler, H., and Skarzynski, B., *Arkiv Kemi, Mineral. Geol.*, **14B**, No. 2, 6 pp. (1940)
155. Skarzynski, B., and von Euler, H., *Arkiv Kemi, Mineral. Geol.*, **14B**, No. 3, 7 pp. (1940)
156. von Euler, H., and Skarzynski, B., *Z. physiol. Chem.*, **265**, 133–46 (1940)
157. Maver, M. E., Johnson, J. M., and Thompson, J. W., *J. Natl. Cancer Inst.*, **1**, 835–43 (1941)
158. Berger, J., Johnson, M. J., and Baumann, C. A., *J. Biol. Chem.*, **137**, 389–95 (1941)
159. Bamann, E., and Schimke, O., *Biochem. Z.*, **310**, 131–51 (1941)
160. Ullmann, E., *Süddeut. Apoth.-Ztg.*, **81**, 521–22 (1941)
161. Boretti, G., *Z. Krebsforsch.*, **52**, 438–42 (1942)
162. Gangl, E., *Z. Krebsforsch.*, **52**, 384–89 (1942)

163. Herken, H., *Z. Krebsforsch.*, **52**, 455–88 (1942)
164. Schmitz, A., *Z. ges. exptl. med.*, **110**, 75–80 (1942)
165. Maschmann, E., *Biochem. Z.*, **315**, 1–25 (1943)
166. Kögl, F., Erxleben, H., and van Veersen, G. J., *Z. physiol. Chem.*, **277**, 251–83 (1943)
167. Kögl, F., *Naturwissenschaften*, **30**, 46–47 (1942)
168. Kögl, F., and Erxleben, H., *Z. physiol. Chem.*, **258**, 57–95 (1939)
169. Graff, S., Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 745–52 (1940)
170. Lipmann, F., Behrens, O. K., Kabat, E. A., and Burk, D., *Science*, **91**, 21–23 (1940)
171. Behrens, O. K., Lipmann, F., Cohn, M., and Burk, D., *Science*, **92**, 32–34 (1940)
172. Kögl, F., Herken, H., and Erxleben, H., *Z. physiol. Chem.*, **264**, 220–39 (1940)
173. Wieland, T., *Ber. deut. chem. Ges.*, **75B**, 1001–7 (1942)
174. Klingmüller, V., *Z. physiol. Chem.*, **278**, 97–119 (1943)
175. Abderhalden, E., *Z. physiol. Chem.*, **275**, 135–54 (1942)
176. Greenstein, J. P., *J. Natl. Cancer Inst.*, **1**, 91–104 (1940)
177. Greenstein, J. P., Thompson, J. W., and Jenrette, W. V., *J. Natl. Cancer Inst.*, **1**, 367–76 (1940)
178. Greenstein, J. P., Jenrette, W. V., and White, J., *J. Natl. Cancer Inst.*, **2**, 305–6 (1941)
179. Dounce, A. L., *J. Biol. Chem.*, **151**, 235–40 (1943)
180. Greenstein, J. P., Turner, F. C., and Jenrette, W. V., *J. Natl. Cancer Inst.*, **1**, 377–85 (1940)
181. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3**, 61–67 (1942)
182. Dittmar, C., *Z. Krebsforsch.*, **52**, 46–56 (1942)
183. Kabat, E. A., *J. Biol. Chem.*, **130**, 143–47 (1939)
184. Ball, H. A., Schott, H. F., and Samuels, L. T., *Cancer Research*, **2**, 146–49 (1942)
185. Dickens, F., and Weil-Malherbe, H., *Cancer Research*, **3**, 73–87 (1943)
186. Dickens, F., *Chemistry & Industry*, **59**, 135 (1940)
187. Dickens, F., *Biochem. J.*, **35**, 1011–23 (1941)
188. Lasnitzki, A., and Brewer, A. K., *Cancer Research*, **1**, 776–78 (1941)
189. Lasnitzki, A., and Brewer, A. K., *Nature*, **149**, 357–58 (1942)
190. Lasnitzki, A., and Brewer, A. K., *Cancer Research*, **2**, 494–96 (1942)
191. Lasnitzki, A., and Brewer, A. K., *Nature*, **142**, 538–39 (1938)
192. Lasnitzki, A., and Brewer, A. K., *Biochem. J.*, **35**, 144–51 (1941)
193. Lasnitzki, A., *Am. J. Cancer*, **35**, 225–29 (1939)
194. Beltrami, W., *Tumori*, **14**, 459–67 (1940)
195. Kahler, K., and Robertson, W. v.B., *J. Natl. Cancer Inst.*, **3**, 495–501 (1943)
196. Burk, D., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 420 (1939)
197. Burk, D., *Symposium on Respiratory Enzymes*, 235–45 (Wisconsin, 1942)
198. Nakatani, M., Nakano, K., and Ohara, Y., *Gann*, **32**, 240–44 (1938)
199. Orr, J. W., and Stickland, L. H., *Biochem. J.*, **35**, 479–87 (1941)

200. Burk, D., Behrens, O. K., and Sugiura, K., *Cancer Research,* **1,** 733–34 (1941)
201. Salter, W. T., Craig, F. N., and Bassett, A. M., *Cancer Research,* **1,** 751 (1941)
202. Craig, F. N., Bassett, A. M., and Salter, W. T., *Cancer Research,* **1,** 869–79 (1941)
203. Roskelley, R. C., Mayer, N., Horwitt, B. N., and Salter, W. T., *J. Clin. Investigation,* **22,** 743–51 (1943)
204. Norris, J. L., Blanchard, J., and Povolny, C., *Arch. Path.,* **34,** 208–17 (1942)
205. Berenblum, I., Chain, E., and Heatley, N. G., *Third Internatl. Cancer Congress,* p. 120 (Atlantic City, N.J., 1939)
206. Berenblum, I., Chain, E., and Heatley, N. G., *Am. J. Cancer,* **38,** 367–71 (1940)
207. Dickens, F., and Weil-Malherbe, H., *Biochem. J.,* **35,** 7–15 (1941)
208. Amersbach, J. C., Walter, E. M., and Cook, E. S., *Arch. Dermatol. Syphilol.,* **46,** 269–75 (1942)
209. Belkin, M., and Stern, K. G., *Cancer Research,* **3,** 164–67 (1943)
210. Burk, D., Sprince, H., Spangler, J. M., Kabat, E. A., Furth, J., and Claude, A., *J. Natl. Cancer Inst.,* **2,** 201–40 (1941)
211. Grant, W. C., and Krantz, J. C., Jr., *Cancer Research,* **2,** 833–36 (1942)
212. Sinai, A. J., *Bull. biol. méd. exptl. U.R.S.S.,* **9,** 230–33 (1940)
213. Burk, D., Sprince, H., Kabat, E. A., and Furth, J., *Cancer Research,* **1,** 732–33 (1941)
214. Burk, D., Sprince, H., Spangler, J. M., Boon, M. C., and Furth, J., *J. Natl. Cancer Inst.,* **3,** 249–75 (1942)
215. Hall, V. E., and Furth, J., *Cancer Research,* **2,** 411–21 (1942)
216. Warren, C. O., *Am. J. Physiol.,* **128,** 455–62 (1940)
217. Warren, C. O., *Am. J. Physiol.,* **131,** 176–86 (1940)
218. Warren, C. O., *Am. J. Physiol.,* **135,** 249–58 (1941)
219. Warren, C. O., *J. Cellular Comp. Physiol.,* **19,** 193–209 (1942)
220. Warren, C. O., *Cancer Research,* **3,** 621–25 (1943)
221. Warren, C. O., and Carter, C. E., *J. Biol. Chem.,* **150,** 267–70 (1943)
222. Warren, C. O., *Federation Proc.,* **2,** 53 (1943)
223. Dickens, F., *Brit. Cancer Campaign, 17th Ann. Rept.,* 135–40 (1939)
224. Dickens, F., *Nature,* **145,** 512–13 (1940)
225. Dickens, F., and Weil-Malherbe, H., *Nature,* **145,** 778–80 (1940)
226. Boyland, E., *Nature,* **145,** 512–13 (1940)
227. Berenblum, I., *Brit. Cancer Campaign, 17th Ann. Rept.,* 214–18 (1939)
228. Berenblum, I., Chain, E., and Heatley, N. G., *Nature,* **145,** 778–79 (1940)
229. Greville, G. D., *Nature,* **148,** 320–21 (1941)
230. Beck, F. F., and Krantz, J. C., Jr., *Cancer Research,* **1,** 188–90 (1941)
231. Crabtree, H. G., *J. Path. Bact.,* **51,** 303–9 (1940)
232. Kensler, C. J., and Rhoads, C. P., *Cancer Research,* **3,** 134–35 (1943)
233. Goldfeder, A., *Cancer Research,* **1,** 220–26 (1941)

234. Feinstein, R. N., and Stare, F. E., *Proc. Soc. Exptl. Biol. Med.*, **45**, 525–29 (1940)
235. Huzita, S., *Japan. J. Obstet. Gynecol.*, **22**, 38–42 (1939)
236. Fardon, J. C., Brotzke, G. C., and Loeffler, M. K., *Studies Inst. Divi Thomae*, **3**, 61–68 (1941)
237. Fardon, J. C., Brotzke, G. C., and Loeffler, M. K., *Studies Inst. Divi Thomae*, **3**, 53–60 (1941)
238. Burk, D., Winzler, R. J., and duVigneaud, V., *J. Biol. Chem.*, **140**, xxi–xxii (1941)
239. Stern, K. G., and Melnick, J. L., *J. Biol. Chem.*, **139**, 301–23 (1941)
240. Engelhardt, V. A., and Sakov, N. C., *Biochimia*, **9**, 9–36 (1943)
241. Sundstroem, E. S., and Michaels, G., *Mem. Univ. Calif.*, **12**, VIII, 1–410 (1942)
242. Rohdenburg, G. L., *Cancer Research*, **1**, 310 (1941)
243. Pollack, M. A., Taylor, A., and Sortomme, C. L., *Cancer Research*, **2**, 828–32 (1942)
244. Allen, F. M., *J. Lab. Clin. Med.*, **26**, 1120–28 (1941)
245. Taylor, A., Thacker, J., and Pennington, D., *Science*, **96**, 342–43 (1942)
246. Taylor, D. R., McAfee, M., and Taylor, A., *Cancer Research*, **3**, 542–45 (1943)
247. Lettre, H., *Angew. Chem.*, **53**, 363–68 (1940)
248. Gemmill, C. L., Gey, G. O., and Austrian, R., *Bull. Johns Hopkins Hosp.*, **66**, 167–84 (1940)
249. Kopac, M. J., Cameron, G., and Chambers, R., *Cancer Research*, **3**, 290–92 (1943)
250. Cameron, G., Kopac, M. J., and Chambers, R., *Cancer Research*, **3**, 281–89 (1943)
251. Brues, A. M., and Jackson, E. B., *Cancer Research*, **1**, 557–63 (1941)
252. Polak, M., *Rev. asoc. med. argentina*, **54**, 31–32 (1940)
253. Fukuoka, F., *Gann*, **35**, 205–7 (1941)
254. Shapiro, C. J., *Am. J. Hyg.*, **31B**, 114–26 (1940)
255. Tennant, R., and Liebow, A. A., *Yale J. Biol. Med.*, **13**, 39–40 (1940)
256. Tennant, R., Liebow, A. A., and Stern, K. G., *Proc. Soc. Exptl. Biol. Med.*, **46**, 18–21 (1940)
257. Vollmar, H., and Inouye, K., *Arch. exptl. Zellforsch. Gewebezücht.*, **23**, 27–41 (1939)
258. Halberstaedter, L., Goldhaber, G., and Doljanski, L., *Cancer Research*, **2**, 28–31 (1942)
259. Doljanski, L., Goldhaber, G., and Halberstaedter, L., *Cancer Research*, **4**, 106–9 (1944)
260. Goldhaber, G., Doljanski, L., and Halberstaedter, L., *Cancer Research*, **4**, 110–12 (1944)
261. Sano, M. E., and Smith, L. W., *Cancer Research*, **2**, 32–39 (1942)
262. Earle, W. R., *J. Natl. Cancer Inst.*, **3**, 555–62 (1943)
263. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3**, 491–94 (1943)
264. Greenstein, J. P., *J. Natl. Cancer Inst.*, **3**, 293–96 (1942)

265. GREENSTEIN, J. P., *J. Natl. Cancer Inst.*, **3**, 287–91 (1942)
266. JONES, H. B., CHAIKOFF, I. L., AND LAWRENCE, J. H., *J. Biol. Chem.*, **128**, 631–44 (1939)
267. JONES, H. B., CHAIKOFF, I. L., AND LAWRENCE, J. H., *J. Biol. Chem.*, **133**, 319–27 (1940)
268. JONES, H. B., CHAIKOFF, I. L., AND LAWRENCE, J. H., *Am. J. Cancer*, **40**, 235–42 (1940)
269. JONES, H. B., CHAIKOFF, I. L., AND LAWRENCE, J. H., *Am. J. Cancer*, **40**, 243–50 (1940)
270. KENNEY, J. M., *Cancer Research*, **2**, 130–45 (1942)
271. KENNEY, J. M., MARINELLI, L. D., AND WOODARD, H. Q., *Radiology*, **37**, 683–87 (1941)
272. KOHMAN, T. P., AND RUSCH, H. P., *Proc. Soc. Exptl. Biol. Med.*, **46**, 403–4 (1941)
273. LAWRENCE, J. H., TUTTLE, L. W., SCOTT, K. G., AND CONNOR, C. L., *J. Clin. Investigation*, **19**, 267–71 (1940)
274. LAWRENCE, J. H., AND SCOTT, K. G., *Proc. Soc. Exptl. Biol. Med.*, **40**, 694–96 (1939)
275. ERF, L. A., AND FRIEDLANDER, G., *Proc. Soc. Exptl. Biol. Med.*, **47**, 134–36 (1941)
276. ERF, L. A., AND LAWRENCE, J. H., *J. Clin. Investigation*, **20**, 567–75 (1941)
277. ERF, L. A., AND LAWRENCE, J. H., *Proc. Soc. Exptl. Biol. Med.*, **46**, 694–95 (1941)
278. ERF, L. A., TUTTLE, L. W., AND LAWRENCE, J. H., *Ann. Internal Med.*, **15**, 487–543 (1941)
279. ERF, L. A., *Am. J. Med. Sci.*, **203**, 529–35 (1942)
280. ERF, L. A., *Proc. Soc. Exptl. Biol. Med.*, **47**, 287–89 (1941)
281. HAMILTON, J. G., *Radiology*, **39**, 541–72 (1942)
282. HEVESY, G., AND VON EULER, H., *Arkiv Kemi, Mineral. Geol.*, **A15**, No. 15, 1–17 (1942)
283. TUTTLE, L. W., ERF, L. A., AND LAWRENCE, J. H., *J. Clin. Investigation*, **20**, 57–61 (1941)
284. TUTTLE, L. W., ERF, L. A., AND LAWRENCE, J. H., *J. Clin. Investigation*, **20**, 577–81 (1941)
285. TUTTLE, L. W., SCOTT, K. G., AND LAWRENCE, J. H., *Proc. Soc. Exptl. Biol. Med.*, **41**, 20–25 (1939)
286. TREADWELL, A., LOW-BEER, B. V. A., FRIEDELL, H. L., AND LAWRENCE, J. H., *Am. J. Med. Sci.*, **204**, 521–30 (1942)
287. WARREN, S., *Cancer Research*, **3**, 334–36 (1943)
288. WARREN, S., *New England J. Med.*, **223**, 751–54 (1940)
289. WARREN, S., AND COWING, R. F., *J. Lab. Clin. Med.*, **26**, 1014–16 (1941)
290. MARSHAK, A., *Science*, **92**, 460–61 (1940)
291. ADLER, E., AND VON EULER, H., *Arkiv Kemi, Mineral. Geol.*, **13A**, No. 26, 14 pp. (1940)
292. JEDLICKA, V., AND SULA, J., *Acta Radiol. Cancerol. Bohemoslov.*, **2**, 108–50 (1939)

293. Lawrence, J. H., Scott, K. G., and Tuttle, L. W., *New Intern. Clin.*, Ser. 2, **3**, 33–58 (1939)
294. Low-Beer, B. V. A., Lawrence, J. H., and Stone, R. S., *Radiology*, **39**, 573–97 (1942)
295. Kenney, J. M., Marinelli, L. D., and Craver, L. F., *Am. J. Roentgenol., Radium Therapy*, **47**, 217–36 (1942)
296. Kenney, J. M., and Craver, L. F., *Radiology*, **39**, 598–607 (1942)
297. Warren, S., *Cancer Research*, **3**, 872–76 (1943)
298. Warren, S., *Cancer Research*, **4**, 113–15 (1944)
299. Abels, J. C., Kenney, J. M., Craver, L. F., Marinelli, L. D., and Rhoads, C. P., *Cancer Research*, **1**, 771–75 (1941)
300. Sugiura, K., *Cancer Research*, **2**, 19–24 (1942)
301. Haven, F. L., *J. Biol. Chem.*, **118**, 111–21 (1937)
302. Haven, F. L., *J. Natl. Cancer Inst.*, **1**, 205–9 (1940)
303. Haven, F. L., *Abstracts Third Intern. Cancer Congr.*, 120–21 (1939)
304. Haven, F. L., and Levy, S. R., *Cancer Research*, **2**, 797–98 (1942)
305. Hunter, F. E., and Levy, S. R., *J. Biol. Chem.*, **146**, 577–81 (1942)
306. Haven, F. L., and Levy, S. R., *J. Biol. Chem.*, **141**, 417–25 (1941)
307. Zahl, P. A., and Cooper, F. S., *Radiology*, **37**, 673–82 (1941)
308. Zahl, P. A., and Waters, L. L., *Proc. Soc. Exptl. Biol. Med.*, **48**, 304–10 (1941)
309. Zahl, P. A., *Surgery*, **9**, 327 (1941)
310. Zahl, P. A., and Cooper, F. S., *Science*, **93**, 64–65 (1941)
311. Moore, F. D., Tobin, L. H., and Aub, J. C., *J. Clin. Investigation*, **22**, 161–68 (1943)
312. Zahl, P. A., Cooper, F. S., and Dunning, J. R., *Proc. Natl. Acad. Sci.*, **26**, 589–98 (1940)
313. Duran-Reynals, F., *Am. J. Cancer*, **35**, 98–107 (1939)
314. Abels, J. C., Ariel, I., Rekers, P. E., Pack, G. T., and Rhoads, C. P., *Arch. Surg.*, **46**, 844–60 (1943)
315. Abels, J. C., Gorham, A. T., Pack, G. T., and Rhoads, C. P., *J. Clin. Investigation*, **20**, 749–64 (1941)
316. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Proc. Soc. Exptl. Biol. Med.*, **54**, 157–58 (1943)
317. Abels, J. C., Gorham, A. T., Pack, G. T., and Rhoads, C. P., *Proc. Soc. Exptl. Biol. Med.*, **48**, 488–92 (1941)
318. Ariel, I., Pack, G. T., and Rhoads, C. P., *Ann. Surg.*, **116**, 924–27 (1942)
319. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Cancer Research*, **4**, 145–48 (1944)
320. Ariel, I., Jones, F., Pack, G. T., and Rhoads, C. P., *Ann. Surg.*, **117**, 740–47 (1943)
321. Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P., *Ann. Internal Med.*, **16**, 221–40 (1942)
322. Abels, J. C., Pack, G. T., and Rhoads, C. P., *Cancer Research*, **3**, 177–79 (1943)
323. Paulson, M., and Wyler, C. I., *Ann. Internal Med.*, **16**, 872–78 (1942)

324. Ariel, I., Abels, J. C., Pack, G. T., and Rhoads, C. P., *Surg. Gynecol. Obstet.*, **77**, 16–25 (1943)
325. Ariel, I., Abels, J. C., Pack, G. T., and Rhoads, C. P., *J. Am. Med. Assoc.*, **123**, 28–30 (1943)
326. Brunschwig, A., Clarke, T. H., Van Prohaska, J., and Schmitz, R., *Ann. Surg.*, **113**, 41–46 (1941)
327. Abels, J. C., Kupel, C. W., Pack, G. T., and Rhoads, C. P., *Cancer Research*, **4**, 145–48 (1944)
328. Minor, A. H., and Ramirez, M. A., *Cancer Research*, **2**, 509–13 (1942)
329. Spellberg, M. A., and Keeton, R. W., *Arch. Internal Med.*, **63**, 1095–116 (1939)
330. Rohdenburg, G. L., *Cancer Research*, **1**, 311–12 (1941)
331. Fasching, H., *Z. ges. exptl. Med.*, **107**, 622–40 (1940)
332. Fasching, H., *Z. ges. exptl. Med.*, **107**, 641–46 (1940)
333. von Euler, H., Säberg, I., and Högberg, B., *Z. physiol. Chem.*, **268**, 171–78 (1941)
334. von Euler, H., Högberg, B., and Säberg, I., *Z. physiol. Chem.*, **274**, 285–90 (1942)
335. Breusch, F. L., *Am. J. Cancer*, **36**, 609–12 (1939)
336. Norris, E. R., and Troescher, E. E., *Cancer Research*, **1**, 410–12 (1941)
337. Bruger, M., and Erlich, S. B., *Arch. Internal Med.*, **72**, 108–14 (1943)
338. Hirschmann, H., *J. Biol. Chem.*, **150**, 363–79 (1943)
339. Smedley-Maclean, I., and Nunn, L. C. A., *Biochem. J.*, **35**, 983–89 (1941)
340. Smedley-Maclean, I., and Hume, E. M., *Biochem. J.*, **35**, 996–1002 (1941)
341. Schins, H. R., and Uelhinger, E., *Z. Krebsforsch.*, **52**, 125–37 (1942)
342. Goeckerman, W. H., and Wilhelm, L. F. X., *Arch. Dermatol. Syphilol.*, **42**, 641–48 (1940)
343. Hueper, W. C., *Cancer Research*, **2**, 551–59 (1942)
344. Nelson, A. A., Fitzhugh, O. G., and Calvery, H. O., *Cancer Research*, **3**, 230–36 (1943)
345. Falin, L. I., and Gromzewa, K. E., *Virchow's Arch. path. Anat.*, **306**, 578–88 (1940)
346. Falin, L. I., and Anissimova, V., *Bull. biol. méd. exptl. U.R.S.S.*, **9**, 518–20 (1940)
347. Andervont, H. B., and Shimkin, M. B., *J. Natl. Cancer Inst.*, **1**, 349–53 (1940)
348. Edwards, J. E., *J. Natl. Cancer Inst.*, **2**, 197–99 (1941)
349. Nettleship, A., and Henshaw, P. S., *J. Natl. Cancer Inst.*, **4**, 309–19 (1944)
350. Bonser, G. M., *J. Path. Bact.*, **54**, 149–54 (1942)
351. Gardner, W. U., *Cancer Research*, **3**, 92–99 (1943)
352. Haerem, A. T., *Proc. Soc. Exptl. Biol. Med.*, **45**, 536–39 (1940)
353. Nelson, A. A., Fitzhugh, O. G., Morris, H. J., and Calvery, H. O., *Cancer Research*, **2**, 11–15 (1942)
354. Nakahara, W., and Mori, K., *Gann*, **35**, 210–33 (1941)

355. Law, L. W., *Proc. Soc. Exptl. Biol. Med.*, **47**, 37–39 (1941)
356. Friedell, H. L., and Rosenthal, L. M., *J. Am. Med. Assoc.*, **116**, 2130–35 (1941)
357. Lorenz, E., Stewart, H. L., Daniel, J. H., and Nelson, C. V., *Cancer Research*, **3**, 123 (1943)
358. Flory, C. M., *Cancer Research*, **1**, 262–76 (1941)
359. Roffo, A. H., *Bol. inst. med. exptl. estud. cáncer*, **18**, 39–68 (1941)
360. Dickens, F., and Weil-Malherbe, H., *Cancer Research*, **2**, 680–84 (1942)
361. Roffo, A. H., *Bol. inst. med. exptl. estud. cáncer*, **17**, 661–98 (1940)
362. Roffo, A. H., *Bol. inst. med. exptl. estud. cáncer*, **18**, 5–38 (1941)
363. Liu, Y., and Hu, C. H., *Proc. Soc. Exptl. Biol. Med.*, **48**, 226–27 (1941)
364. Roffo, A. H., *Bol. inst. med. exptl. estud. cáncer*, **17**, 385–414 (1940)
365. Beck, S., *Brit. J. Exptl. Path.*, **22**, 299–302 (1941)
366. Pollia, J. A., *J. Ind. Hyg. Toxicol.*, **23**, 449–53 (1941)
367. Berenblum, I., *Cancer Research*, **1**, 44–48 (1941)
368. Berenblum, I., *Cancer Research*, **1**, 807–14 (1941)
369. Sall, R. D., Shear, M. J., Leiter, J., and Perrault, A., *J. Natl. Cancer Inst.*, **1**, 45–55 (1940)
370. Steiner, P. E., *Cancer Research*, **2**, 181–82 (1942)
371. Woglom, W. H., *Am. J. Cancer*, **40**, 429–30 (1940)
372. Campbell, J. A., *Brit. Med. J.*, **2**, 275–80 (1940)
373. Campbell, J. A., *Brit. Med. J.*, **1**, 217–21 (1942)
374. McDonald, S., Jr., and Woodhouse, D. L., *J. Path. Bact.*, **54**, 1–12 (1942)
375. Leiter, J., and Shear, M. J., *J. Natl. Cancer Inst.*, **3**, 164–74 (1942)
376. Leiter, J., Shimkin, M. B., and Shear, M. J., *J. Natl. Cancer Inst.*, **3**, 155–65 (1942)
377. Pessano, J. E., *Semana med. (Buenos Aires)*, **II**, 210–14 (1942)
378. Franseen, C. C., Aub, J. C., and Simpson, C. L., *Cancer Research*, **1**, 393–96 (1941)
379. Fieser, L. F., *Am. J. Cancer*, **34**, 37–124 (1938)
380. Fieser, L. F., *Cause and Growth of Cancer*, pp. 1–27 (Univ. Penna. Bicentennial Conf., Univ. Penna. Press, Philadelphia, 1941)
381. Cook, J. W., *Ergeb. Vitamin Hormoneforsch.*, **2**, 213–58 (1939)
382. Cook, J. W., and Kennaway, E. L., *Am. J. Cancer*, **39**, 381–428, 521–82 (1940)
383. Badger, G. M., Cook, J. W., Hewett, C. L., Kennaway, E. L., Kennaway, N. M., Martin, R. H., and Robinson, A. M., *Proc. Roy. Soc. (London), B.*, **129**, 439–67 (1940)
384. Cook, J. W., and Martin, R. H., *J. Chem. Soc.*, 1125–27 (1940)
385. Cook, J. W., Hewett, C. L., Kennaway, E. L., and Kennaway, N. M., *Am. J. Cancer*, **40**, 62–77 (1940)
386. Cook, J. W., *Chemistry & Industry*, **60**, 242–43 (1941)
387. Everett, J. L., and Hewett, C. L., *J. Chem. Soc.*, 1159–62 (1940)
388. Dunlap, C. E., and Warren, S., *Cancer Research*, **1**, 953–54 (1941)
389. Shear, M. J., Leiter, J., and Perrault, A., *J. Natl. Cancer Inst.*, **1**, 303–35 (1940)

390. Shear, M. J., Leiter, J., and Perrault, A., *J. Natl. Cancer Inst.*, **2**, 99–112 (1941)
391. Shear, M. J., and Leiter, J., *J. Natl. Cancer Inst.*, **2**, 241–58 (1941)
392. Law, L. W., and Lewisohn, M., *Cancer Research*, **1**, 695–98 (1941)
393. Bergmann, W., *Z. Krebsforsch.*, **48**, 546–52 (1939)
394. Bruce, W. F., *J. Am. Chem. Soc.*, **63**, 304–5 (1941)
395. Bowen, E. J., *Nature*, **149**, 528 (1942)
396. Weil-Malherbe, H., and Weiss, J., *Nature*, **149**, 471–72 (1942)
397. Jones, R. N., *J. Am. Chem. Soc.*, **63**, 151–55 (1941)
398. Bernstein, S., Kauzmann, W. J., and Wallis, E. S., *J. Org. Chem.*, **6**, 319–30 (1940)
399. Weiss, J., *Nature*, **147**, 512 (1941)
400. Wood, J., and Fieser, L. F., *J. Am. Chem. Soc.*, **63**, 2323–31 (1941)
401. Creech, H. J., and Jones, R. N., *J. Am. Chem. Soc.*, **62**, 1970–75 (1940)
402. Creech, H. J., and Jones, R. N., *J. Am. Chem. Soc.*, **63**, 1661–69 (1941)
403. Creech, H. J., and Jones, R. N., *J. Am. Chem. Soc.*, **63**, 1670–73 (1941)
404. Peacock, P. R., *Am. J. Cancer*, **40**, 251–54 (1940)
405. Davenport, H. A., Savage, J. L., Dirstine, M. J., and Queen, F. B., *Cancer Research*, **1**, 821–24 (1941)
406. Dickens, F., and Weil-Malherbe, H., *Cancer Research*, **2**, 560–66 (1942)
407. Davis, W. M., Krahl, M. E., and Clowes, G. H. A., *J. Am. Chem. Soc.*, **64**, 108–10 (1942)
408. Shear, M. J., *J. Natl. Cancer Inst.*, **3**, 455–77 (1943)
409. Lorenz, E., Shimkin, M. B., and Stewart, H. L., *J. Natl. Cancer Inst.*, **1**, 355–60 (1940)
410. Rusch, H. P., Kline, B. E., and Baumann, C. A., *Cancer Research*, **2**, 183–88 (1942)
411. Mider, G. B., and Morton, J. J., *J. Natl. Cancer Inst.*, **1**, 41–44 (1940)
412. Lauridsen, J., and Eggers, H. E., *Cancer Research*, **3**, 43–46 (1943)
413. Auerbach, C., *Proc. Roy. Soc. Edinburgh*, **60**, 164–73 (1940)
414. Kensler, C. J., *Symposium on Respiratory Enzymes*, 246–51 (Univ. of Wisconsin Press, 1942)
415. Kensler, C. J., Dexter, S. O., and Rhoads, C. P., *Cancer Research*, **2**, 1–10 (1942)
416. Kensler, C. J., Young, N. F., and Rhoads, C. P., *J. Biol. Chem.*, **143**, 465–72 (1942)
417. Potter, V. R., *Cancer Research*, **2**, 688–93 (1942)
418. Kuhn, R., and Beinert, H., *Ber. deut. chem. Ges.*, **76**, 904–8 (1943)
419. Deutsch, H. F., Kline, B. E., and Rusch, H. P., *J. Biol. Chem.*, **141**, 529–38 (1941)
420. Deutsch, H. F., Miner, D. L., and Rusch, H. P., *Cancer Research*, **1**, 818–20 (1941)
421. Warren, F. L., *Chemistry & Industry*, **61**, 170 (1942)
422. Rusch, H. P., and Kline, B. E., *Cancer Research*, **1**, 465–72 (1941)
423. Wasley, W. L., and Rusch, H. P., *Cancer Research*, **2**, 422–24 (1942)
424. Mueller, G. C., and Rusch, H. P., *Cancer Research*, **3**, 113–16 (1943)

425. Miller, J. A., Kline, B. E., Rusch, H. P., and Baumann, C. A., *Cancer Research,* **4,** 153–58 (1944)
426. Cramer, W., and Stowell, R. E., *J. Natl. Cancer Inst.,* **2,** 369–78 (1942)
427. Cramer, W., and Stowell, R. E., *J. Natl. Cancer Inst.,* **2,** 379–402 (1942)
428. Cowdry, E. V., *Biol. Symposia,* **X,** 131–62 (The Jaques Cattell Press, Lancaster, Pa., 1943)
429. Cowdry, E. V., and Paletta, F. X., *Am. J. Path.,* **17,** 335–58 (1941)
430. Cowdry, E. V., and Paletta, F. X., *J. Natl. Cancer Inst.,* **1,** 745–59 (1941)
431. Cooper, Z. K., and Reller, H. C., *J. Natl. Cancer Inst.,* **2,** 335–44 (1942)
432. Wicks, L. F., and Suntzeff, V., *J. Natl. Cancer Inst.,* **3,** 221–26 (1942)
433. Stowell, R. E., *J. Natl. Cancer Inst.,* **3,** 111–21 (1942)
434. Suntzeff, V., and Carruthers, C., *Cancer Research,* **3,** 431–33 (1943)
435. Carruthers, C., and Suntzeff, V., *Cancer Research,* **3,** 744–48 (1943)
436. Carruthers, C., and Suntzeff, V., *J. Natl. Cancer Inst.,* **3,** 217–20 (1942)
437. Aivasian, A. I., *Bull. biol. méd. exptl. U.R.S.S.,* **9,** 521–23 (1940)
438. Hamperl, H., Graffi, A., and Longer, E., *Z. Krebsforsch.,* **53,** 133–84 (1942)
439. Earle, W. R., *J. Natl. Cancer Inst.,* **4,** 165–212 (1943)
440. Earle, W. R., and Nettleship, A., *J. Natl. Cancer Inst.,* **4,** 213–28 (1943)
441. Nettleship, A., and Earle, W. R., *J. Natl. Cancer Inst.,* **4,** 229–48 (1943)
442. Nettleship, A., *J. Natl. Cancer Inst.,* **3,** 559–62 (1943)
443. Larionow, L. T., Chertkova, M. S., and Samokhvalova, A. S., *Bull. biol. méd. exptl. U.R.S.S.,* **9,** 515–17 (1940)
444. Burk, D., and MacNeary, D. F., *Record of Chemical Progress,* **5,** 28–32 (1944).
445. Taylor, A., *Science,* **97,** 123 (1943)
446. Taylor, A., *Southern Med. Surg.,* **105,** 291–95 (1943)
447. Taylor, A., Hungate, R. E., and Taylor, D. R., *Cancer Research,* **3,** 537–41 (1943)
448. Bittner, J. J., *Cancer Research,* **2,** 710–21 (1942)
449. Bittner, J. J., *Trans. Coll. Physicians Phila.,* **9,** 129–43 (1943)
450. Bittner, J. J., *J. Natl. Cancer Inst.,* **1,** 155–68 (1940)
451. Bittner, J. J., *Proc. Soc. Exptl. Biol. Med.,* **45,** 805–10 (1940)
452. Bittner, J. J., *Cancer Research,* **4,** 159–67 (1944)
453. Bittner, J. J., *U.S. Pub. Health Repts.,* **54,** 1827–30 (1939)
454. Bittner, J. J., *Science,* **93,** 527–28 (1941)
455. Bittner, J. J., *Science,* **95,** 462–63 (1942)
456. Woolley, G. W., Law, L. W., and Little, C. C., *Cancer Research,* **1,** 955–56 (1941)
457. Andervont, H. B., Shimkin, M. B., and Bryan, W. R., *J. Natl. Cancer Inst.,* **3,** 309–18 (1942)
458. Bryan, W. R., Kahler, H., Shimkin, M. B., and Andervont, H. B., *J. Natl. Cancer Inst.,* **2,** 451–55 (1942)
459. Visscher, M. B., Green, R. G., and Bittner, J. J., *Proc. Soc. Exptl. Biol. Med.,* **49,** 94–96 (1942)
460. Andervont, H. B., *J. Natl. Cancer Inst.,* **2,** 307–8 (1941)
461. Fekete, E., and Little, C. C., *Cancer Research,* **2,** 525–30 (1942)

462. ANDREWES, C. H., *Proc. Roy. Soc. Med.*, **33**, 78–86 (1939)
463. LAW, L. W., *Science*, **93**, 381–82 (1941)
464. LAW, L. W., *Cancer Research*, **2**, 108–15 (1942)
465. BARNES, W. A., *Cancer Research*, **1**, 99–101 (1941)
466. FURTH, J., COLE, R. K., AND BOON, M. C., *Cancer Research*, **2**, 280–83 (1942)
467. KIRSCHBAUM, A., AND STRONG, L. C., *Proc. Soc. Exptl. Biol. Med.*, **51**, 404–6 (1942)
468. STEINER, P. E., *Surg. Gynecol. Obstet.*, **76**, 105–12 (1943)
469. KLEINENBERG, H. E., NEUFACH, S. A., AND SHABAD, L. M., *Am. J. Cancer*, **39**, 463–88 (1940)
470. KLEINENBERG, H. E., NEUFACH, S. A., AND SHABAD, L. M., *Cancer Research*, **1**, 853–59 (1941)
471. SHABAD, L. M., *Compt. rend. soc. biol.*, **124**, 213–16 (1937)
472. STEINER, P. E., *Science*, **92**, 431–32 (1940)
473. STEINER, P. E., *Cancer Research*, **2**, 425–35 (1943)
474. STEINER, P. E., *Proc. Soc. Exptl. Biol. Med.*, **51**, 352–53 (1942)
475. STEINER, P. E., *Cancer Research*, **3**, 385–95 (1943)
476. SANNIE, C., TRUHAUT, R., AND GUERIN, P., *Bull. assoc. franc. étude cancer*, **29**, 107–21 (1941)
477. VINCENTI, M., *Arch. ital. Chir.*, **62**, 381–94 (1942)
478. APTEKMAN, P. M., KING, H. D., AND LEWIS, M. R., *Cancer Research*, **3**, 856–57 (1943)
479. DES LIGNERIS, M. J. A., *Am. J. Cancer*, **39**, 489–95 (1940)
480. HIEGER, I., *Am. J. Cancer*, **39**, 496–503 (1940)
481. HIEGER, I., *Science*, **93**, 262–300 (1941)
482. MENKE, J. F., *Science*, **92**, 290–91 (1940)
483. MENKE, J. F., *Cancer Research*, **2**, 786–93 (1942)
484. STEELE, R., KOCH, F. C., AND STEINER, P. E., *Cancer Research*, **1**, 614–17 (1941)
485. BOWMAN, R. O., AND MOTTSHAW, H. R., *Cancer Research*, **1**, 308–9 (1941)
486. SOBOTKA, H., AND BLOCH, E., *Am. J. Cancer*, **35**, 50–54 (1939)
487. TURNER, F. C., *U.S. Public Health Repts.*, **54**, 1603–9 (1939)
488. PENN, H. S., *Nature*, **149**, 193–94 (1942)
489. PENN, H. S., *J. Chem. Phys.*, **10**, 145–46 (1942)
490. HIEGER, I., *Nature*, **149**, 300–1 (1942)
491. MENKIN, V., *Cancer Research*, **1**, 548–56 (1941)
492. MILLER, F. R., AND HAUSE, W. A., *Proc. Soc. Exptl. Biol. Med.*, **45**, 387–88 (1940)
493. MILLER, F. R., HAUSE, W. A., AND JONES, H. W., *Proc. Soc. Exptl. Biol. Med.*, **50**, 115–16 (1942)
494. TURNER, D. L., AND MILLER, F. R., *J. Biol. Chem.*, **147**, 573–79 (1943)
495. DRUCKREY, H., RICHTER, R., AND VIERTHALER, R., *Klin. Wochschr.*, **20**, 781–85 (1941)
496. HASHIMOTO, T., *Gann*, **29**, 306–8 (1935)
497. STEVENSON, E. S., DOBRINER, K., AND RHOADS, C. P., *Cancer Research*, **2**, 160–67 (1942)

498. JACOBI, H. P., AND BAUMANN, C. A., *Cancer Research*, **2**, 175–80 (1942)
499. WHITE, J., AND WHITE, A., *J. Biol. Chem.*, **131**, 149–61 (1939)
500. WHITE, J., *J. Natl. Cancer Inst.*, **1**, 337–41 (1940)
501. WHITE, J., AND EDWARDS, J. E., *J. Natl. Cancer Inst.*, **2**, 531–33 (1942)
502. WOOD, J. L., AND FIESER, L. F., *J. Am. Chem. Soc.*, **62**, 2674–81 (1940)
503. CHALMERS, J. G., AND PEACOCK, P. R., *Biochem. J.*, **30**, 1242–48 (1936)
504. CHALMERS, J. G., *Biochem. J.*, **32**, 271–78 (1938)
505. CHALMERS, J. G., *Biochem. J.*, **34**, 678–84 (1940)
506. CHALMERS, J. G., AND KIRBY, A. H. M., *Biochem. J.*, **34**, 1191–95 (1940)
507. PEACOCK, P. R., *Brit. J. Exptl. Path.*, **17**, 164–72 (1936)
508. CHALMERS, J. G., AND CROWFOOT, D., *Biochem. J.*, **35**, 1270–75 (1941)
509. BERENBLUM, I., AND SCHOENTAL, R., *Nature*, **149**, 439–40 (1942)
510. BERENBLUM, I., AND SCHOENTAL, R., *Biochem. J.*, **36**, 92–97 (1942)
511. BERENBLUM, I., AND SCHOENTAL, R., *Cancer Research*, **3**, 145–50 (1943)
512. BERENBLUM, I., CROWFOOT, D., HOLIDAY, E. R., AND SCHOENTAL, R., *Cancer Research*, **3**, 151–58 (1943)
513. MOTTRAM, J. C., AND WEIGERT, F., *Nature*, **150**, 635 (1942)
514. DONIACH, I., MOTTRAM, J. C., AND WEIGERT, F., *Brit. J. Exptl. Path.*, **24**, 1–9 (1943)
515. DONIACH, I., MOTTRAM, J. C., AND WEIGERT, F., *Brit. J. Exptl. Path.*, **24**, 9–14 (1943)
516. WEIGERT, F., AND MOTTRAM, J. C., *Biochem. J.*, **37**, 497–501 (1943)
517. BOYLAND, E., AND LEVI, A. A., *Biochem. J.*, **30**, 1225–27 (1936)
518. BOYLAND, E., LEVI, A. A., MAWSON, E. H., AND ROE, E., *Biochem. J.*, **35**, 184–91 (1941)
519. DOBRINER, K., RHOADS, C. P., AND LAVIN, G. I., *Proc. Soc. Exptl. Biol. Med.*, **41**, 67–69 (1939)
520. DOBRINER, K., RHOADS, C. P., AND LAVIN, G. I., *Cancer Research*, **2**, 95–107 (1942)
521. CASON, J., AND FIESER, L. F., *J. Am. Chem. Soc.*, **62**, 2681–87 (1940)
522. CASON, J., AND FIESER, L. F., *J. Am. Chem. Soc.*, **63**, 1256–58 (1941)
523. JONES, R. N., *Cancer Research*, **2**, 245–51 (1942)
524. JONES, R. N., *Cancer Research*, **2**, 252–55 (1942)
525. BERENBLUM, I., AND SCHOENTAL, R., *Cancer Research*, **3**, 686–90 (1943)
526. CHANG, L. H., AND YOUNG, L., *Proc. Soc. Exptl. Biol. Med.*, **53**, 126–29 (1943)
527. CHANG, L. H., AND YOUNG, L., *J. Biol. Chem.*, **151**, 87–91 (1943)
528. CHANG, L. H., *J. Biol. Chem.*, **151**, 93–99 (1943)
529. CHALMERS, J. G., AND PEACOCK, P. R., *Biochem. J.*, **35**, 1276–82 (1941)
530. LORENZ, E., AND SHIMKIN, M. B., *J. Natl. Cancer Inst.*, **2**, 491–98 (1942)
531. GYÖRGY, P., TOMARELLI, R., OSTERGARD, R. P., AND BROWN, J. B., *J. Exptl. Med.*, **76**, 413–20 (1942)
532. ANDERVONT, H. B., *J. Natl. Cancer Inst.*, **1**, 361–66 (1940)
533. SHEAR, M. J., *Cancer Research*, **1**, 731–32 (1941)
534. HARTWELL, J. L., SHEAR, M. J., AND ADAMS, J. R., *Cancer Research*, **3**, 122 (1943)
535. SHEAR, M. J., AND TURNER, F. C., *J. Natl. Cancer Inst.*, **4**, 81–97 (1943)

536. SHEAR, M. J., PERRAULT, A., AND ADAMS, J. R., *J. Natl. Cancer Inst.*, **4**, 99–105 (1943)
537. HARTWELL, J. L., SHEAR, M. J., AND ADAMS, J. R., *J. Natl. Cancer Inst.*, **4**, 107–22 (1943)
538. KAHLER, H., SHEAR, M. J., AND HARTWELL, J. L., *J. Natl. Cancer Inst.*, **4**, 123–29 (1943)
539. ZAHL, P. A., HUTNER, S. H., SPITZ, S., SUGIURA, K., AND COOPER, F. S., *Am. J. Hyg.*, **36**, 224–42 (1942)
540. ZAHL, P. A., AND HUTNER, S. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 116–18 (1943)
541. ZAHL, P. A., AND HUTNER, S. H., *Proc. Soc. Exptl. Biol. Med.*, **51**, 285–87 (1942)
542. ZAHL, P. A., HUTNER, S. H., AND COOPER, F. S., *Proc. Soc. Exptl. Biol. Med.*, **54**, 48–50 (1943)
543. HUTNER, S. H., AND ZAHL, P. A., *Science*, **96**, 563–64 (1942)
544. HUTNER, S. H., AND ZAHL, P. A., *Proc. Soc. Exptl. Biol. Med.*, **52**, 364–68 (1943)
545. LAWRENCE, E. A., AND DURAN-REYNALS, F., *Yale J. Biol. Med.*, **14**, 177–81 (1941)
546. SHWARTZMAN, G., *Cancer Research*, **4**, 191–96 (1944)
547. ANDERVONT, H. B., AND SHIMKIN, M. B., *Am. J. Cancer*, **36**, 451–59 (1939)
548. SHIMKIN, M. B., AND ZON, L., *J. Natl. Cancer Inst.*, **3**, 379–82 (1943)
549. BOYLAND, E., AND BOYLAND, M. E., *Biochem. J.*, **31**, 454–60 (1937)
550. BRUES, A. M., MARBLE, B. B., AND JACKSON, E. B., *Am. J. Cancer*, **38**, 159–68 (1940)
551. HIRSHFELD, J. W., TENNANT, R., AND OUGHTERSON, A. W., *Yale J. Biol. Med.*, **13**, 51–59 (1940)
552. LEWISOHN, R., LEUCHTENBERGER, R., AND LASZLO, D., *Surg. Gynecol. Obstet.*, **71**, 274–85 (1940)
553. BARRETT, M. K., *J. Natl. Cancer Inst.*, **2**, 625–30 (1942)
554. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., LASZLO, D., AND BLOCH, K., *Cancer Research*, **1**, 799–806 (1941)
555. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., AND LASZLO, D., *Am. J. Path.*, **17**, 251–60 (1941)
556. LEWISOHN, R., LASZLO, D., LEUCHTENBERGER, C., LEUCHTENBERGER, R., AND DISCHE, Z., *Proc. Soc. Exptl. Biol. Med.*, **52**, 269–72 (1943)
557. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., LASZLO, D., AND DISCHE, Z., *Proc. Soc. Exptl. Biol. Med.*, **52**, 272–73 (1943)
558. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., LASZLO, D., AND BLOCH, K., *Science*, **94**, 70–71 (1941)
559. LASZLO, D., AND LEUCHTENBERGER, C., *Cancer Research*, **3**, 401–10 (1943)
560. LEWISOHN, R., LEUCHTENBERGER, C., LEUCHTENBERGER, R., LASZLO, D., AND DISCHE, Z., *Cancer Research*, **2**, 818–22 (1942)
561. LEWISOHN, R., LASZLO, D., LEUCHTENBERGER, C., LEUCHTENBERGER, R., AND DISCHE, Z., *Proc. Soc. Exptl. Biol. Med.*, **52**, 269–72 (1943)
562. LASZLO, D., AND LEUCHTENBERGER, C., *Science*, **97**, 515 (1943)
563. BOYLAND, E., *Biochem. J.*, **35**, 1283–88 (1941)

564. Boyland, E., *Chemistry & Industry,* **60,** 219–20 (1941)
565. Dobrovolskaïa-Zavadskaïa, N., and Zephiroff, P., *Compt. rend. soc. biol.,* **134,** 60–63 (1940)
566. Dobrovolskaïa-Zavadskaïa, N., and Zephiroff, P., *Compt. rend. soc. biol.,* **134,** 79–81 (1940)
567. Dobrovolskaïa-Zavadskaïa, N., and Zephiroff, P., *Compt. rend. soc. biol.,* **133,** 216–18 (1940)
568. Dobrovolskaïa-Zavadskaïa, N., and Zephiroff, P., *Compt. rend. soc. biol.,* **133,** 391–94 (1940)
569. Rohdenburg, G. L., and Nagy, S. M., *Am. J. Cancer,* **29,** 66–77 (1937)
570. Thompson, J. H., Holt, P. F., Jones, R. F., Haydu, N., and Kennedy, G. Y., *Med. Press & Circ.,* **205,** 334–42 (1941)
571. Turner, F. C., *U.S. Pub. Health Repts.,* **54,** 1855–63 (1939)
572. Rowntree, L. C., and Ziegler, W. M., *Proc. Soc. Exptl. Biol. Med.,* **54,** 121–23 (1943)
573. Beard, H. H., *Arch. Biochem.,* **1,** 177–86 (1942)
574. Beard, H. H., *Exptl. Med. Surg.,* **1,** 123–35 (1943)
575. Beard, H. H., *Exptl. Med. Surg.,* **1,** 136–42 (1943)
576. Hammett, F. S., *Proc. Soc. Exptl. Biol. Med.,* **45,** 601–2 (1940)
577. Hammett, F. S., *Growth,* **5,** 69–83 (1941)
578. Hammett, F. S., *Growth,* **5,** 85–111 (1941)
579. Strong, L. C., *Proc. Soc. Exptl. Biol. Med.,* **43,** 634–37 (1940)
580. Strong, L. C., *Cancer Research,* **1,** 473–76 (1941)
581. Boyland, E., *Biochem. J.,* **34,** 1196–1201 (1940)
582. Garai, F., *Cancer Research,* **1,** 144–45 (1941)
583. Carruthers, C., *Arch. Path.,* **30,** 1184–91 (1940)
584. Long, M. L., and Bischoff, F., *Am. J. Cancer,* **38,** 406–7 (1940)
585. Carruthers, C., and Stowell, R. E., *Cancer Research,* **1,** 724–28 (1941)
586. Belkin, M., *Cancer Research,* **2,** 264–68 (1942)
587. Belkin, M., *Cancer Research,* **2,** 269–75 (1942)
588. Belkin, M., *Cancer Research,* **2,** 276–79 (1942)
589. Kline, B. E., Wasley, W. L., and Rusch, H. P., *Cancer Research,* **2,** 645–48 (1942)
590. Kline, B. E., and Rusch, H. P., *Cancer Research,* **3,** 702–5 (1943)
591. Tanaka, A., and Tuboi, S., *Gann,* **34,** 354–55 (1940)
592. Tuboi, S., *Mitt. med. Akad. Keijo,* **32,** 420 (1941)
593. Turner, F. C., *J. Natl. Cancer Inst.,* **4,** 265–70 (1943)
594. Badger, G. M., Elson, L. A., Haddow, A., Hewett, C. L., and Robinson, A. M., *Proc. Roy. Soc. (London),* **B130,** 255–99 (1942)
595. Carr, J. G., *Brit. J. Exptl. Path.,* **23,** 221–28 (1942)
596. Turner, F. C., *U.S. Pub. Health Repts.,* **54,** 1279–88 (1939)
597. Stamer, S., *Acta Path. Microbiol. Scand.,* **20,** Suppl. 4, 1–158 (1943)
598. Ruffilli, D., *Boll. soc. ital. biol. sper.,* **17,** 75–77 (1942)
599. DuBilier, B., and Warren, S. L., *Cancer Research,* **1,** 966–69 (1941)
600. Bloch, H., *Arch. ges. Virusforsch.,* **1,** 481–96 (1940)
601. Michell, F., *Chem.-Ztg.,* **66,** 55–56 (1942)
602. Ball, H. A., *Cancer Research,* **2,** 823–27 (1942)

603. Thompson, J. H., Holt, P. F., and Callow, H. J., *Nature*, **151**, 364–65 (1943)
604. Popoff, M., *Z. Krebsforsch.*, **52**, 32–36 (1942)
605. Crabtree, H. G., *Cancer Research*, **1**, 34–38 (1941)
606. Crabtree, H. G., *Cancer Research*, **1**, 39–43 (1941)
607. Braun-Stappenbeck, M., *Süddeut. Apoth.-Ztg.*, **82**, 203–5 (1942)
608. Flory, C. M., Furth, J., Saxton, J. A., Jr., and Reiner, L., *Cancer Research*, **3**, 729–43 (1943)
609. Stamer, S., and Holm, J. E., *Acta Path. Microbiol. Scand.*, **20**, 360–71 (1943)
610. Laurence, W. L., *Science*, **94**, 88–89 (1941)
611. du Vigneaud, V., Spangler, J. M., Burk, D., Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Science*, **95**, 174–76 (1942)
612. Burk, D., Spangler, J. M., du Vigneaud, V., Kensler, C. J., Sugiura, K., and Rhoads, C. P., *Cancer Research*, **3**, 130 (1943)
613. Kaplan, I., Zurrow, M., and Goldfeder, A., *Abstracts of 28th Meeting of the Radiological Society*, p. 43 (Chicago, Illinois, Nov. 30–Dec. 4, 1942)
614. Kaplan, I., *Am. J. Med. Sci.* (In press)
615. Rhoads, C. P., and Abels, J. C., *J. Am. Med. Assoc.*, **121**, 1261–63 (1943)
616. Kensler, C. J., Wadsworth, C., Sugiura, K., Rhoads, C. P., Dittmer, K., and du Vigneaud, V., *Cancer Research*, **3**, 823–24 (1943)
617. Schade, A. L., and Caroline, L., *Science* (In press)
618. Meyer, K., *Science*, **99**, 391–92 (1944)
619. Laurence, W. L., *Science*, **99**, 392–93 (1944)
620. Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, **99**, 203–5 (1944)
621. Lilly, V. G., and Leonian, L. H., *Science*, **99**, 205–6 (1944)

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THE ALKALOIDS

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The period under review has been productive of a fair amount of progress in alkaloid research in spite of the unsettled condition of world affairs. Most of the progress has, however, come from English speaking countries. The large volume of work coming from continental Europe and to a lesser extent from the Orient in normal times is largely lacking or not readily accessible for review. Our South American neighbors are beginning to explore the excessively rich alkaloid reserves of their continent, and it is hoped that the next decade will witness many important contributions from there.

No attempt will be made to catalogue the many new alkaloids which have been reported, but it should be pointed out that their isolation is the necessary preliminary to their further investigation. It is therefore desirable to isolate as many new alkaloids as possible since progress in their further investigation is generally more rapid when a number of related alkaloids are available. The study of an individual alkaloid then is not an isolated project but a coordinated portion of a much larger and perhaps unified field. It is well known that alkaloids may be divided into a limited number of structural types, and in many cases it is possible to recognize the type to which an alkaloid belongs by elementary and functional group analyses supplemented by color tests. The determination of structure in some such cases is then almost a matter of routine. An outstanding example of this thesis is represented by the protoberberine alkaloids and their near relatives, the protopine type bases. Members of one group are convertible into those of the other, and it is a fact that the elucidation of the structure of any individual would have been more difficult had it not been for the occurrence of both types and many representatives of each type. Specifically, the synthesis of allocryptopine might not yet have been accomplished if it could not have been synthesized from berberine. The writer therefore considers it of prime importance to isolate as many new alkaloids as possible. Many will be recognized as belonging to well known types and the elucidation of their structures will be rendered easier by this knowledge. Others, however, will belong to hitherto unrecognized types, and their structural analysis

will be greatly simplified if many examples are available. In this connection it is pertinent to point out that very little progress in the study of the Papillionaceous alkaloids was made until the structure of cytisine was settled for once and all. Some members of a type are more amenable to chemical examination than others, and the knowledge gained from one can often be extrapolated to others. But here a word of caution is timely. Although the alkaloids contained in any particular plant, and often in an entire botanical family, are generally related structurally, they are not necessarily so. The copresence of anabasine, a pyridine base, and aphylline and lupinine, Papillionaceous alkaloids, in *Anabasis aphylla* is sufficient warning. Incidentally, the plant belongs to a family (Chenopodiaceae) which had not, until then, been regarded as capable of elaborating alkaloids. To add to the dilemma, salsoline was found to be a simple isoquinoline alkaloid, and yet its source, *Salsola richteri,* also belongs to the Chenopodiaceae. It is becoming commonplace to find the same alkaloid in plants of widely separated botanical families and no reasonable taxonomic concessions could be expected to bring these families into closer relation. Not only does nicotine occur in *Nicotiana* and *Duboisia* (Solanaceae) but it has recently been found in *Asclepias* (Asclepiadaceae) (1), *Equisetum* (Equisetaceae) (2) and *Lycopodium* (Lycopodiaceae) (2). These families actually belong to three different botanical orders and they are furthermore separated by the prime distinctions of Phanerogams and Cryptogams. It is certain that nicotine is not typically related to solanidine (3) and perhaps other Solanaceous alkaloids. It would therefore be presumptuous to expect it to be typically related to all other alkaloids with which it occurs, although its close relation to the other tobacco alkaloids has been ascertained. It would appear as though nicotine, and perhaps a few other alkaloids, are elaborated by a mechanism which is present, either latent or active, in virtually all plants.

Lycopodium alkaloids.—The fact that *Lycopodium* species contain alkaloids has long been a matter of record (4) but the first really concise research in this field is one by Achmatowicz & Uzieblo (5) who isolated three alkaloids from *Lycopodium clavatum* L. of European origin. They retained the name lycopodine for the chief alkaloid. This name is particularly appropriate because lycopodine seems to be the one alkaloid which is present in nearly all Lycopodium species although Deulefeu & DeLanghe (6) have not recorded it as present in *L. saururus* of South American origin. The writer, in

collaboration with Marion (2, 7, unpublished) has isolated lycopodine from the following Canadian Lycopodiums: *L. complanatum, L. annotinum, L. clavatum, L. obscurum, L. lucidulum,* and *L. tristachyum.* As congeners to lycopodine some fifteen new alkaloids have been found. Most of them, like lycopodine ($C_{16}H_{25}ON$) contain only one nitrogen atom but the number of oxygen atoms varies from none to three. In general they contain either sixteen or eighteen carbon atoms, and the premise is that they are closely related structurally. Some, however, contain two nitrogen atoms and their relation to lycopodine is more problematical.

When lycopodine was heated with selenium it yielded 7-methyl- and 5:7-dimethylquinoline, and in small experiments the former was obtained by heating the alkaloid with palladium and with phthalic anhydride (8). While it is possible that deep seated structural changes may have been effected by these none too mild treatments, it is assumed at present that a fully reduced quinoline nucleus forms the basis of the lycopodine molecule. Complanatine, one of the congeners of lycopodine in *L. complanatum,* has also yielded 7-methylquinoline in a small experiment. On the other hand annotinine ($C_{16}H_{21}O_3N$) from *L. annotinum* does not yield a recognizable quinoline derivative when heated with selenium. This does not necessarily mean that it is not structurally related to lycopodine because it contains an additional two oxygen atoms, which are present in a lactone ring, and some of the carbon atoms of this ring may also be part of the quinoline nucleus.

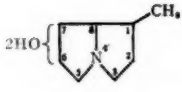
Some of the above work has not yet been published. The work on *L. clavatum* also remains to be published, and it is of interest in this connection that clavatine and clavatoxine (5) isolated from the European plant could not be obtained and are in fact not present. The significance of this observation is not yet clear. Taxonomists regard the European and American species as identical. There remains the disconcerting possibility that varietal distinctions or habitat have a profound influence on the nature of the elaborated alkaloids.

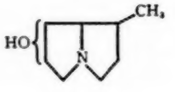
Senecio alkaloids.—The family Compositae is one of the largest recognized, but in general it is notoriously poor in alkaloids. The lone exception is the genus *Senecio,* and closely related or perhaps synonymous genera, *Erechtites,* which comprises well over a thousand species and most of these contain alkaloids. The first species examined, *S. latifolius,* yielded two alkaloids (9) one of which was shown to be an alkamine ester. The basic moiety resulting from alka-

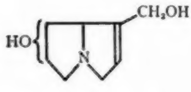
line hydrolysis was given the formula $C_8H_{11}O_2N$, but later work indicated that it was probably identical with retronecine (10, 11), which was obtained by the hydrolysis of retrorsine obtained from *S. retrorsus*. Subsequently retronecine was recognized as the basic constituent of a large number of *Senecio* and other alkamine ester alkaloids not only by the writer but by others (12 to 15). In a few isolated cases only was retronecine replaced by its dihydro- or anhydro-derivative. Some progress toward the elucidation of the structure of retronecine was made when Orechoff and co-workers recognized that it is closely related to the basic fragment heliotridine, of lasiocarpine, itself an alkamine ester obtained from *Heliotropium lasiocarpum* (Boraginaceae). Heliotridine ($C_8H_{13}ON$) was deoxygenated by successive treatment with phosphorus pentachloride and reduction; and the resulting base, heliotridane ($C_8H_{15}N$) was shown to be identical with a base similarly obtained from retronecine.

The greatest progress was made in the study of retronecine when Adams and co-workers (12) discovered that monocrotoline from *Crotalaria spectabilis* (Leguminosae) on hydrolysis also yields retronecine which then became available in amounts adequate for research.

Adams and co-workers have revised their earlier formula (17) for retronecine on the basis of new experimental evidence. Retronecine on reduction with hydrogen over Raney nickel yields platynecine so that the latter is dihydroretronecine. The earlier work of Men'shikov (18) had indicated that platynecine is dihydroxy-1-methylpyrrolidizine (I) and this was confirmed. Catalytic reduction of retronecine yielded retronecanol (II) as the result of hydrogenolysis of the

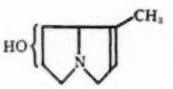

I


II

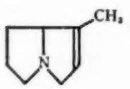

III

hydroxyl group which was recognized as primary. A C-methyl determination on II was positive whereas retronecine gave a negative result. That retronecine is not a derivative of vinylamine was inferred from a study of a number of vinylamines so that the 2:3-position for the double bond is unlikely. The 1:2-position was chosen

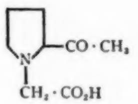
because easy hydrogenolysis is a characteristic of α,β-unsaturated alcohols. Conclusive proof of the position of the double bond was obtained as follows (19): desoxyretronecine (IV) was converted into the corresponding chloro-compound by treatment with thionyl chloride and then reduced with chromous chloride to isoheliotridene (V). The last was subjected to ozonolysis in aqueous solution and the



IV

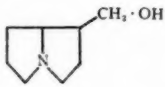


V

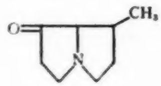


VI

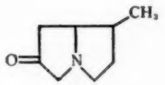
product was regarded as 2-acetyl-l-pyrrolidine-acetic acid (VI). It was shown to contain the $CH_3 \cdot CO$-group and a carboxyl, and it yielded a lactone. No other position of the double bond could yield a compound containing the same groups. It is therefore obvious that retronecine can be represented by III. Benzoyl-platynecine when converted to the chloro-derivative and reduced in the presence of Raney nickel yielded a base, isoretronecanol which must be formulated as VII and which could be oxidized to the corresponding carboxylic acid.



VII



VIII

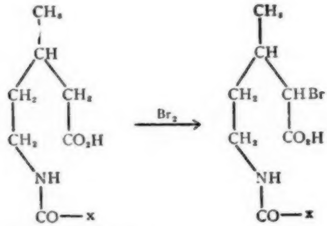


IX

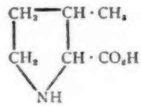
The nature of the second hydroxyl group as secondary was shown by oxidation of retronecanol with cyclohexanone and aluminum tertiary butoxide to a ketone, retronecanone, which must therefore be either VIII or IX since it yields an oxime and is still basic.

While this review was in preparation Professor Roger Adams has kindly acquainted the writer with a manuscript which has been accepted for publication and will shortly appear in the *Journal of the American Chemical Society*. In collaboration with Leonard he has succeeded in synthesizing retronecanone as follows: N-*m*-Nitroben-

zoyl-4-methylpiperidine was oxidized to δ-*m*-nitrobenzoylamino-β-methylvaleric acid (X), the acid chloride of which on bromination yielded the corresponding α-bromo-acid (XI). This on hydrolysis and treatment with alkali underwent ring closure to yield a methyl proline of formula XII. When the ethyl ester was treated with ethyl acrylate

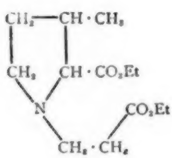


X. x=*m*-nitrophenyl   XI

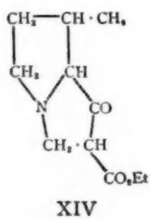


XII

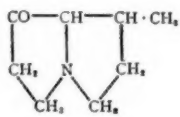
there was obtained a di-ester of formula XIII which on ring closure with metallic potassium yielded a cyclic ketonic ester (probably XIV but the alternate possibility would be equally suitable), acid hydrolysis of which resulted in *dl*-retronecanone (XV). When the above series



XIII



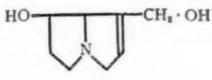
XIV



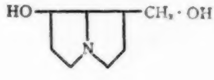
XV

of reactions was carried out with optically inactive X the final product XV could conceivably consist of four stereoisomeric substances and attempts at resolution failed. The entire series of reactions was therefore repeated after X had been resolved into its optically active components by means of quinidine. The retronecanone (XV) thus obtained from the *l*-acid (X) proved to be identical with the degradative base. It is therefore possible to write unambiguous formulae for

retronecine (XVI), platynecine (XVII), and the various derivatives and degradation products.



XVI



XVII

The following table is a summary of most of the known alkamine ester bases which give retronecine or a derivative on hydrolysis.

Alkaloid	Source	Base	Acid
Senecionine $C_{18}H_{25}O_5N$	*Senecio vulgaris* (26, 27) *S. viscosus* (27) *S. squalidus* (27) *S. aureus* (14, 28, 29) *S. ilicifolius* (16) *S. jacobaea* (10) *S. pseudo-arnica* (28) *S. integerrimus* (28)	Retronecine $C_8H_{13}O_2N$	Senecic $C_{10}H_{14}O_4$
Jacobine $C_{18}H_{25}O_6N$	*S. jacobaea* (10, 14, 15) *S. cineraria* (14) *S. erucifolia* (15)	Retronecine	Jaconecic $C_{10}H_{16}O_6$
Seniciphylline $C_{18}H_{23}O_5N$	*S. platyphyllus* (24, 25) *S. spartioides* (28) *S. stenocephallus* (24, 25)	Retronecine	Seneciphyllic $C_{10}H_{14}O_5$
Retrorsine $C_{18}H_{25}O_6N$	*S. retrorsus* (10) *S. isatideus* (15) *S. graminifolius* (16) *S. glaberrimus* (15) *S. pterophorus* (16) *S. venosus* (15) *S. ilicifolius* (16)	Retronecine	Retronecic lactone
Senecifoline	*S. latifolius* (11, 22)	Senecifolinine $C_8H_{11}O_2N$	Senecifolic $C_{10}H_{16}O_6$
Hieracifoline $C_{18}H_{25}O_5N$	*Erechtites hieracifolia* (29)	Retronecine	Hieracinecic
Integerrimine $C_{18}H_{25}O_5N$	*Senecio integerrimus* (28)	Retronecine	Integerrinecic $C_{10}H_{16}O_5$
Longilobine $C_{18}H_{23}O_5N$	*S. longilobus* (28)	Retronecine	Longinecic $C_{10}H_{14}O_5$
Spartioidine $C_{18}H_{23}O_5N$	*S. spartioides* (28)		

Alkaloid	Source	Base	Acid
Riddelline $C_{18}H_{23}O_6N$	*S. riddellii* (28, 32)	Retronecine	Riddellic $C_{10}H_{14}O_6$
Platyphylline $C_{18}H_{25}O_5N$	*S. platyphyllus* (13, 24)	Platynecine $C_8H_{15}O_2N$	Senecic $C_{10}H_{14}O_4$
Mikanoidine $C_{21}H_{29}O_6N$	*S. mikanioides* (26)	Mikanecine $C_8H_{15}O_2N$	Mikanecic $C_{13}H_{16}O_5$
Platyphylline $C_{18}H_{25}O_5N$	*S. platyphyllus* (13); *S. adnatus*	Platynecine $C_8H_{15}O_2N$	Senecic
Graminifoline $C_{18}H_{23}O_5N$	*S. graminifolius* (16)	?	?
Pterophine $C_{18}H_{23}O_5N$	*S. pterophorus* (16); *S. ilicifolius* (16)	Retronecine	Pterophnecic lactone $C_{10}H_{16}O_6$
Isatidine $C_{18}H_{27}O_7N$	*S. isatideus* (20, 21); *S. retrorsus* (20, 21)	Isatinecine $C_8H_{13}O_3N$	Isatinecic R·CO·O·OH
Otosenine $C_{19}H_{27}O_7N$	*S. othonnae* (22)	Otonecine $C_9H_{15}O_3N$	Jaconecic
Rosmarinine $C_{18}H_{27}O_6N$	*S. rosmarinifolius* (23); *S. hygrophilus* (66); *S. brachypodus* (66); *S. pauciligulatus* (66)	Rosmarinecine $C_8H_{13}O_3N$	Senecic
Monocrotalline $C_{16}H_{23}O_6N$	*Crotalaria spectabilis* (12); *C. retusa* (12)	Retronecine	Monocrotic $C_7H_{12}O_3$
Grantianine $C_{18}H_{23}O_7N$	*C. grantiana* (30)	Retronecine	
Heliotrine $C_{16}H_{27}O_5N$	*Heliotropium lasiocarpum* (33)	Heliotridine $C_8H_{15}O_2N$	Heliotrinic $C_8H_{16}O_4$
Lasiocarpine $C_{21}H_{33}O_7N$	*H. lasiocarpum* (33)	Heliotridine $C_8H_{15}O_2N$	Lasiocarpinic $C_8H_{16}O_5$ and angelic
Trachelanthine $C_{15}H_{25}O_5N$	*Trachelanthus korolkovi* (31)	$C_8H_{13}O_2N$	$C_7H_{14}O_4$
Trochodesmine $C_{18}H_{27}O_6N$	*Trichodesma incanum* (13)	Retronecine	*dl*-lactic and keto-acid

Papillionaceous alkaloids.—The last decade has witnessed remarkable progress in the difficult field of the Papillionaceous alkaloids. The constitutions of cytisine, lupinine, and *dl*-oxy-sparteine have been satisfactorily determined, the last two having been synthesized as well. The structures of several others have been determined in whole or in

part and a number have been shown to be amine oxides. This is the only family of plants which is known to elaborate amine oxides although the presence of trimethylamine oxide in fish muscle is a well known fact. A number of new alkaloids have been reported (34). Worthy of special comment however is the first recorded natural occurrence of 3-methoxypyridine not only in *Thermopsis rhombifolia* (Leguminosae) but also in *Equisetum arvense* (Equisetaceae) (35). The role of this, one of the simplest natural alkaloids, and the nature of its precursors in plant metabolism remain a subject for speculation.

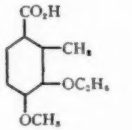
Papaveraceous alkaloids.—The writer has published a series of papers dealing with plants of the Papaveraceae and particularly of the Fumariaceae. The latter has been treated as a sub-family by Fedde (36) in Engler's *Pflanzenreich* but Hutchinson (37) finds sufficient taxonomic evidence to retain two families. It seems however that as long as two families are retained the position of a number of genera will be in dispute, and the classification will then be a matter of personal preference. It may be permissible to quote Hutchinson verbatim.

> *Fumariaceae* is now generally recognised as a family distinct from the *Papaveraceae* proper. It is noteworthy, however, that in both Bentham and Hooker's Genera Plantarum, and Engler and Prantl's Pflanzenfamilien, it stands as a subfamily and tribe respectively of *Papaveraceae.* As a separate family it was first described by A. P. DeCandolle in his "Systema" (1821), again in the "Prodromus" (1824), and by Lindley in his "Vegetable Kingdom" (1853). Endlicher (1836) ranked the group as a subfamily. The present writer is not so much concerned as to whether the *Fumariaceae* should be regarded as a family or a subfamily. In his opinion it is quite distinctly separated as a group from *Papaveraceae* proper, and nearly as closely allied to certain genera of *Berberidaceae,* such as *Epimedium, Aceranthus* and *Bongardia.* That there is close affinity with certain *Papaveraceae* is quite evident, especially with *Chelidonium* and allied genera. But it is probable that this alliance is more apparent than real, and that the *Fumariaceae* have not arisen directly from the ancestors of the present *Papaveraceae.*

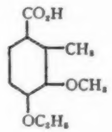
The writer would like to add that the alliance is more real than apparent, and it is his opinion that the nature of the contained alkaloids can already decide the issue. No plant in the entire Papaveraceae family has yet been found to be devoid of alkaloids and at least one alkaloid, namely protopine, is present in every plant. What is equally significant is that protopine has never been found in any plants of other families. There is some taxonomic doubt as to whether or not the recently established genus, *Oceanopapaver,* should be relegated to Papaveraceae at all. If the plant should become available (it is native

to New Caledonia) a chemical examination would certainly resolve the question. The probability that protopine is actually present in all Papaveraceous plants is becoming more certain in view of its presence in a number of hitherto unexamined genera, namely, *Hypecoum procumbens, Hypecoum leptocarpum, Dendromecon rigida, Platycapnos spicatus, Meconopsis, Cysticapnos vesicarius* (unpublished). The plants named were available in small amounts only but it can be stated that protopine is the major constituent. The congeners of protopine, in those plants which have been adequately examined, and where their constitutions are known, are without exception isoquinoline alkaloids. There are however a great many types of isoquinoline alkaloids and the different types vary in the frequency of their occurrence. Cryptopine and allocryptopine are not restricted to a few genera. (The latter has been isolated from *Xanthoxylon brachyacanthum*, a Rutaceae.) The phthalide-isoquinoline alkaloids (narcotine, bicuculline, adlumine, etc.) are confined to *Papaver, Corydalis, Dicentra,* and *Adlumia,* although hydrastine occurring in *Hydrastis canadensis* (Berberidaceae) also belongs to this type. There is however a distinction between the *Papaver* phthalide-isoquinolines and those of other genera. Narcotine has been found only in the former. It is distinct from the other phthalide-isoquinolines in that it has five alkoxy groups, the others having only four. The protoberberine bases (typified by berberine, palmatine, and their tetrahydro-derivatives) are botanically the widest distributed of all isoquinoline alkaloids. In addition to Papaveraceae they are present in Berberidaceae, Rutaceae, and Ranunculaceae but not in the genus *Papaver*. In fact they appear to be restricted to *Corydalis, Dicentra, Fumaria, Stylophorum,* and *Dicranostigma* (38). The aporphine bases are restricted but to other genera, namely, *Corydalis, Dicentra, Glaucium,* and *Roemeria*. They also occur in other families (Menispermaceae, Lauraceae, Anonaceae) but in many cases they lack the N-methyl group or contain less than four alkoxy groups and are more often than not phenolic. The benzylisoquinoline bases occur exclusively in *Papaver* whereas the phenanthridine bases (chelidonine, sanguinarine, etc.) are confined to a group of closely related genera, namely, *Chelidonium, Stylophorum, Dicranostigma, Sanguinaria, Bocconia* (39), and *Macleaya*. However, the closely related *Hunnemannia* is devoid of them but contains the first example of a desmethyl base, hunnemanine, in the protopine series. The structure of hunnemanine was shown to be that of one of the two possible 0-desmethyl allocryptopines since on methylation with diazo-

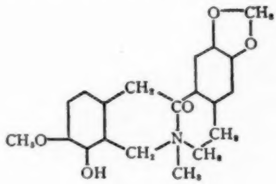
methane it yielded the latter (40). The 0-ethyl ether was degraded by the well known Perkin procedure and the methoxy-ethoxy-*o*-toluic acid finally obtained must be either XVIII or XIX. It seemed likely



XVIII



XIX



XX

that the sterically hindered position would carry the free hydroxyl group and a synthetic specimen of XVIII proved to be identical with the acid obtained from the alkaloid. Hunnemanine therefore has formula XX.

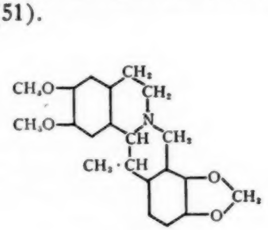
It is the writer's opinion that the facts already enumerated are sufficient to justify the conclusion that there is no longer any reason for retaining separate family distinction for Papaveraceae and Fumariaceae. When attempts are made to reclassify genera and species within a genus, the results are not so concise. This is due in part to the limited number of plants examined, and to the fact that the constitutions of many of the alkaloids have not yet been determined. Some conclusions can nevertheless be drawn. At one time the genus *Stylophorum* included what is now known as *Dicranostigma*. Chemically there is no reason for such differentiation since the three alkaloids (chelidonine, protopine, and stylopine) which occur in *S. diphyllum* also occur in *D. franchetianum* (38). Species in these genera (or this genus) are few, and it is hoped that the examination of a third species, *D. lactucoides*, will be possible shortly.

As a matter of taxonomic convenience and orderliness it has been found desirable to divide polytypic genera into sections and subsections. Such divisions are often admittedly arbitrary but the credulous are inclined to regard them as fundamental. Ultimately the characters, chemical as well as morphological, of a plant must depend upon genetic fundamentals. A chromosome analysis cannot be decisive, and a complete study of the genes is not yet possible. However a chemical analysis does not depend upon preconceived ideas of classification so that when taxonomists and chemists fail to agree the latter have the

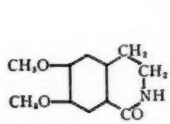
advantage. Fortunately agreement is much more common than disagreement. A large number of plants of the section *Eucorydalis* have been examined. Aside from three exceptions, namely, *C. sempervirens* (41), *C. crystallina* (42), and *C. incisa* (unpublished), the alkaloids contained in the plants are structurally closely related (43 to 48) and this relation extends in many cases to the minor alkaloids. The exceptions noted are devoid of bisisoquinoline alkaloids, but contain instead phthalide-isoquinolines. The conclusion is inescapable that the close taxonomic relation between the above three species and the other members of the section *Eucorydalis* is no more than accidental.

Progress in determining the constitutions of the many new alkaloids has been necessarily slow, partly because of shortage of material, and partly because it has not been possible to relegate the alkaloids to specific types. The constitutions of only twelve alkaloids out of the 50 to 60 new ones isolated by the author and collaborators have as yet been determined. The recent work on cryptocavine (49) is a reminder that new types or radical variations of recognized types of isoquinoline alkaloids are still to be looked for.

The recent investigation of a *Corydalis* which belongs to a section of the genus not hitherto examined is a reminder that many new alkaloids still await discovery although the genus *Dactylicapnos* proved disappointing (50). *Corydalis thalictrifolia* (Sub-section Thalictrifoliae) yielded a new alkaloid, thalictrifoline, which on demethylenation, methylation of the dihydric phenol, and racemization yielded *dl*-corydaline. Oxidation yielded 1-keto-6:7-dimethoxy-tetrahydroisoquinoline XXII, and therefore the structure is that represented by XXI (51).



XXI



XXII

Veratrum and solanum alkaloids.—Soltys & Wallenfels (3) were the first to adduce positive evidence that solanidine was structurally related to the sterols. Selenium degradation yielded Diel's hydro-

carbon (methylcyclopentenophenanthrene) and the base itself gave a digitonide. Craig & Jacobs (52) obtained 2-ethyl-5-methylpyridine from protoveratrine, cevine, jervine, and also from solanidine by selenium degradation. Recently they (53) showed that rubijervine and isorubijervine gave precipitates with digitonin and confirmed the earlier work of Soltys & Wallenfels that solanidine on treatment with selenium yields Diel's hydrocarbon. They also obtained from the reaction mixture small amounts of 2-methyl- and 2:3-dimethylphenanthrene. Although it is possible to write detailed structural formulae on the basis of the above and other observations, such formulae are not convincing, except in so far as they contain the carbon skeleton of the sterols.

Erythrina alkaloids.—Although no noteworthy advances have been recorded in determining the constitutions of the Erythrina alkaloids an important advance in their isolation has been recorded. It was found that, after the alkaloids had been removed from an alkaline solution by solvent extraction, a large portion of the characteristic physiological activity still resided in the aqueous solution. Acid (preferably) or alkaline hydrolysis followed by solvent extraction of the slightly alkaline hydrolyzate yielded new types of bases which were termed the eryso-alkaloids. The chief of these is erysopine, $C_{17}H_{19}O_3N$ (one methoxyl group) which was shown to occur in the seeds of ten Erythrina species (54). Erysonine is isomeric with erysopine (55). Erysodine, erysocine, and erysonine are isomeric and represented by $C_{18}H_{21}O_3N$ containing two methoxyl groups.

Miscellaneous alkaloids.—The phenolic alkaloid magnoline from *Magnolia fuscata* has been shown to be 7:7′-desmethyldauricine (56). Dauricine has also been isolated from *Menispermum canadense* and its constitution confirmed (57).

Heliotrinic acid, the acidic fission product of heliotrine, has been shown to be α-hydroxy-α-isopropyl-β-methoxy-butyric acid (58).

Petrosimonia monandra (Chenopodiaceae) has yielded 1.33 per cent of piperidine (59). The authors claim that it is the first recorded natural occurrence, but Rimington had shown (60) as early as 1934 that the hydrochloride could be obtained in 4.5 per cent yield from *Psilocaulon absimile.*

Another Chenopodiaceae, *Girgensohnia diptera,* has yielded an alkaloid termed dipterine (61). It was shown to be identical with *N*-methyltryptamine. Still another Chenopodiaceae, *Arthrophytum leptocladum,* also contained dipterine as well as *N*-methyl-β-phenyl-

ethylamine and a new base termed leptocladine (62, 63). The last was shown to be 2:3-dimethyltetrahydrocarboline, a formula already suggested by Barger *et al.* (64) for calycanthidine. The latter, however, is optically active whereas leptocladine and the synthetic base are not, so that the Barger formula may still be correct.

The natural occurrence of pyridine to the extent of 2 per cent is recorded for the first time from a plant, namely, *Aplopappus hartwegi* (65). Other bases are also present.

LITERATURE CITED

1. Marion, L., *Can. J. Research*, **B17**, 21–22 (1939)
2. Manske, R. H. F., and Marion, L., *Can. J. Research*, **B20**, 87–92 (1942)
3. Soltys, A., and Wallenfels, K., *Ber. deut. chem. Ges.*, **69**, 811–18 (1936)
4. Bödeker, K., *Ann.*, **208**, 363–67 (1881)
5. Achmatomicz, C., and Uzieblo, W., *Roczniki Chem.*, **18**, 88–95 (1938)
6. Deulefeu, N., and DeLanghe, J., *J. Am. Chem. Soc.*, **64**, 968–69 (1942)
7. Manske, R. H. F., and Marion, L., *Can. J. Research*, **21B**, 92–96 (1943)
8. Marion, L., and Manske, R. H. F., *Can. J. Research*, **20B**, 153–56 (1942)
9. Watt, H. E., *J. Chem. Soc.*, **95**, 466–77 (1909)
10. Manske, R. H. F., *Can. J. Research*, **5**, 651–59 (1931)
11. Barger, G., Seshadri, T. R., Watt, H. E., and Yabuta, T., *J. Chem. Soc.*, 11–15 (1935)
12. Adams, R., and Rogers, E. F., *J. Am. Chem. Soc.*, **61**, 2815–19 (1939)
13. Konovalova, R., and Orekhov, A., *Bull. soc. chim.* (5), **4**, 2037–42 (1937)
14. Barger, G., and Blackie, J. J., *J. Chem. Soc.*, 584–86 (1937)
15. Blackie, J. J., *Pharm. J.*, **138**, 102–4 (1937)
16. deWaal, H. L., *Onderstepoort J. Vet. Sci. Animal Ind.*, **16**, 149–66 (1941)
17. Adams, R., and Rogers, E. F., *J. Am. Chem. Soc.*, **63**, 228–35, 537–41 (1941)
18. Men'shikov, G. P., *Bull. acad. sci. U.R.S.S.*, 969 (1936)
19. Adams, R., and Mahan, J. E., *J. Am. Chem. Soc.*, **65**, 2009–12 (1943)
20. deWaal, H. L., *Onderstepoort J. Vet. Sci. Animal Ind.*, **14**, 433–47 (1940)
21. deWaal, H. L., *Onderstepoort J. Vet. Sci. Animal Ind.*, **12**, 155–63 (1939)
22. Zhdanovich, E. S., and Men'shikov, G. P., *J. Gen. Chem. (U.S.S.R.)*, **4**, 835–38 (1941)
23. deWaal, H. L., *Nature*, **146**, 177–78 (1940)
24. Orekhov, A., and Tiedebel, W., *Ber. deut. chem. Ges.*, **68**, 650–55 (1935)
25. Orekhov, A., Konovalova, R., and Tiedebel, W., *Ber. deut. chem. Ges.*, **68**, 1886–90 (1935)
26. Manske, R. H. F., *Can. J. Research*, **14B**, 5–11 (1936)
27. Barger, G., and Blackie, J. J., *J. Chem. Soc.*, 743–45 (1936)
28. Manske, R. H. F., *Can. J. Research*, **17B**, 1–7 (1939)
29. Manske, R. H. F., *Can. J. Research*, **17B**, 8–9 (1939)
30. Adams, R., Carmack, M., and Rogers, E. F., *J. Am. Chem. Soc.*, **64**, 571–73 (1942)
31. Men'shikov, G. P., and Borodina, G. M., *J. Gen. Chem. (U.S.S.R.)*, **11**, 209–12 (1941)
32. Adams, R., Hamlin, K. E., Jr., Jelnick, C. F., and Phillips, R. F., *J. Am. Chem. Soc.*, **64**, 2760–63 (1942)
33. Men'shikov, G. P., *J. Gen. Chem. (U.S.S.R.)*, **9**, 1851–55 (1939)
34. Manske, R. H. F., and Marion, L., *Can. J. Research*, **21B**, 144–48 (1943)
35. Manske, R. H. F., *Can. J. Research*, **20B**, 265–67 (1942)
36. Fedde, F., *Die Natürlichen Pflanzenfamilien* (Engler-Prantl, W. Engelmann, Leipzig, 1936)
37. Hutchinson, J., *Kew Bulletin*, 97–115 (1921)
38. Manske, R. H. F., *Can. J. Research*, **20B**, 53–56 (1942)
39. Manske, R. H. F., *Can. J. Research*, **21B**, 140–43 (1943)

40. Manske, R. H. F., Marion, L., and Ledingham, A. E., *J. Am. Chem. Soc.*, **64**, 1659–61 (1942)
41. Manske, R. H. F., *Can. J. Research*, **8**, 407–11 (1933)
42. Manske, R. H. F., *Can. J. Research*, **17B**, 57–60 (1939)
43. Manske, R. H. F., *Can. J. Research*, **9**, 436–42 (1933)
44. Manske, R. H. F., *Can. J. Research*, **16B**, 81–90 (1938)
45. Manske, R. H. F., *Can. J. Research*, **16B**, 153–57 (1938)
46. Manske, R. H. F., *Can. J. Research*, **18B**, 80–83 (1940)
47. Manske, R. H. F., *Can. J. Research*, **20B**, 49–52 (1942)
48. Manske, R. H. F., *Can. J. Research*, **20B**, 265–67 (1942)
49. Manske, R. H. F., and Marion, L., *J. Am. Chem. Soc.*, **62**, 2042–44 (1940)
50. Manske, R. H. F., *Can. J. Research*, **21B**, 117–18 (1943)
51. Manske, R. H. F., *Can. J. Research*, **21B**, 111–16 (1943)
52. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **143**, 427–32 (1942)
53. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **149**, 451–64 (1943)
54. Folkers, K., and Koniuszy, F., *J. Am. Chem. Soc.*, **62**, 1677–83 (1940)
55. Folkers, K., Shavel, J., Jr., and Koniuszy, F., *J. Am. Chem. Soc.*, **63**, 1544–49 (1941)
56. Proskurnina, N. F., and Orekhov, A. P., *Bull. soc. chim.* (5), **5**, 1357–60 (1938)
57. Manske, R. H. F., *Can. J. Research*, **21B**, 17–20 (1943)
58. Men'shikov, G. P., *J. Gen. Chem. (U.S.S.R.)*, **9**, 1851–55 (1939)
59. Yurashevskiĭ, N. K., and Stepanov, J. I., *J. Gen. Chem. (U.S.S.R.)*, **9**, 1687–89 (1939)
60. Rimington, C., *S. African J. Sci.*, **31**, 184–93 (1934)
61. Yurashevskiĭ, N. K., *J. Gen. Chem. (U.S.S.R.)*, **10**, 1781–83 (1940)
62. Yurashevskiĭ, N. K., *J. Gen. Chem. (U.S.S.R.)*, **9**, 595–97 (1939)
63. Yurashevskiĭ, N. K., *J. Gen. Chem. (U.S.S.R.)*, **11**, 157–62 (1941)
64. Barger, G., Jacob, A., and Madinaveitia, J., *Rec. trav. chim.*, **57**, 548–54 (1938)
65. Buehrer, T. F., Mason, T. F., and Crowder, J. A., *Am. J. Pharm.*, **111**, 105–12 (1939)
66. Richardson, M. F., and Warren, F. L., *J. Chem. Soc.*, 452–54 (1943)

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SYNTHETIC DRUGS—ANTISPASMODICS

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At the present time, the most spectacular accomplishments in the field of synthetic drugs are represented by the discoveries of certain chemotherapeutic agents, such as the sulfonamides, and of relatively simple compounds, i.e., stilbestrol and vitamin-K substitutes, which can be employed to advantage clinically in place of certain hormones or vitamins. These achievements have been truly remarkable ones, especially in view of the elusive and little-understood nature of the relationship between chemical structure and pharmacological activity, and their practical value can hardly be overestimated.

It would be unfortunate, however, if research activities were limited to attempts to discover only products which, because of their highly dramatic effects, promote wide-spread interest. The alleviation of pain and suffering, through the administration of such drugs as anesthetics, hypnotics, analgesics, pressor agents, and antispasmodics, is still as important as ever in the practice of medicine. Innovations in these fields which contribute to a greater margin of safety and a greater range of therapeutic usefulness are most desirable.

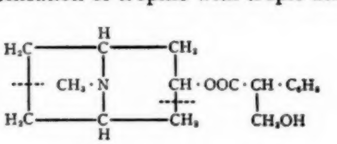
Antispasmodics

While some synthetic drugs were discovered solely as the result of fortuitous circumstances, others would hardly be known at the present time if it were not for our knowledge of certain medicaments of plant origin. To a large extent, synthetic antispasmodics owe their development to the two naturally occurring drugs, atropine and papaverine. The chemical structures of these latter compounds have served as patterns for the synthesis of potent products which resemble their plant prototypes in molecular architecture; however, other active synthetic compounds have been discovered in which structural similarity to the original plant drugs is hardly discernible.

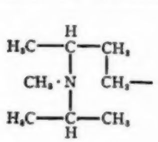
Before a discussion of individual compounds is begun, it seems pertinent to mention more in detail the manner in which knowledge of the structure of a given pharmacologically active plant compound may be utilized to supply clues to the structures of new compounds which, if synthesized, might be expected to exhibit pharmacological

activity and, possibly, possess properties superior to those of the plant drug.

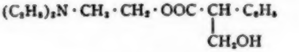
The ester, atropine or 3-tropoyltropine (I), represents the condensation of tropine with tropic acid. An examination of the alcoholic



I



II



III

radical of atropine shows that it is characterized by a completely saturated bicyclic structure and by the presence of a strongly basic nitrogen atom. The cyclic nucleus creates a certain "bulk" in the molecule and, furthermore, in this instance makes *cis-trans* stereoisomeric forms possible. If these two features are disregarded—although it should be emphasized that in specific instances they may play an important role in connection with the pharmacological potency of a molecule—the alcoholic radical in tropine may be considered merely as a complex basic-alkyl group.

With this point of view the question arises, could simpler basic-alkyl groups be substituted for the tropyl radical without loss of activity and, if so, what groups?

If the ring structures were opened at the points shown by the dotted lines in formula I, and four hydrogen atoms were added, the tropane ring would be converted into the acyclic, basic-alkyl radical γ-(methylisopropylamino)-γ-methylpropyl (II). Such a radical, in the form of the corresponding alcohol, cannot be synthesized too readily; hence, the preparation of an ester which contained this nucleus as a part of its structure would be somewhat difficult. However, by the use of more readily available alcohols such as γ-dimethylamino-propanol or β-diethylaminoethanol, products which contain the simpler but analogous basic-alkyl groups, γ-dimethylaminopropyl or β-diethylaminoethyl, can be obtained.

Preparation and pharmacological examination, then, of an ester (III) prepared from β-diethylaminoethanol, instead of tropine, and

tropic acid might prove that this simpler substance exhibits the desired type of activity. In this event, a number of similar esters would be synthesized. The possible variations of such a compound as that represented by formula III are so numerous that it is impractical to prepare all of them.

After it has been determined whether or not other radicals can be substituted for the tropyl group without loss of activity, attention would be directed to the acidic group in the atropine molecule. Tropic acid is α-phenyl-β-hydroxypropionic acid or phenyl-(hydroxymethyl)-acetic acid. For a reason which will become apparent later, we prefer the latter name since it indicates that tropic acid is a disubstituted acetic acid. At least some of the many isomers, homologues, and analogues, which may be used in place of tropic acid for the preparation of esters, readily suggest themselves.

When the complex basic alcoholic radical (2-carbmethoxytropyl) in cocaine, 3-benzoyl-2-carbmethoxytropine, is replaced by a simple basic alkyl group, and *p*-aminobenzoyloxy is substituted for benzoyloxy, a type of local anesthetic is obtained which reaches a high peak in usefulness in procaine (β-diethylaminoethyl *p*-aminobenzoate). It was this fact which led investigators to believe that effective antispasmodics would be discovered among esters in which both the alcoholic and the acyl radicals were different from those present in atropine.

A number of synthetic analogues of atropine have been described in the literature. The two features of these products which interest us are their structure and their antispasmodic activity. There is no uncertainty with regard to the former, but many difficulties present themselves when attempts are made to establish the latter in a strictly quantitative sense. In order that the origin and nature of these difficulties may be better understood, it seems advisable to state very briefly the manner in which the pharmacological data are obtained, how they are reported, and how they are to be interpreted.

The customary procedure is to determine, first, the activity of a new compound on the isolated strip of rabbit intestine by the method of Magnus (1, 2). After a kymograph tracing of the normal, rhythmic movements of the muscle has been obtained, the compound to be tested is introduced into the bath which surrounds the strip, and the kymograph record then shows very clearly whether or not relaxation has been produced. However, since the object in the use of an antispasmodic is not so much to effect relaxation in the normal muscle as to

achieve this state in the muscle when it is in a state of spasm, evaluation of the substance on the untreated intestine often is not determined. A spastic state is induced in the intestinal muscle by the introduction into the bath of a very small amount of barium chloride or acetylcholine. It is recognized that smooth muscle spasm may be caused either by direct stimulation of the muscle (musculotropic) or by indirect stimulation through the nerve endings (neurotropic); hence, a potential antispasmodic must be tested for effectiveness against both types of spasm. Barium chloride is a musculotropic, acetylcholine a neurotropic stimulant.

The very marked differences between the effectiveness of papaverine and that of atropine against spastic conditions is illustrated by the experimental data in Table I. It can be seen that the amount of papaverine, expressed as maximum effective dilution or minimum effective dose, required to relax the intestinal strip after stimulation with barium chloride is much less than the quantity of atropine necessary to effect the same degree of relaxation. Since papaverine counteracts the spasm produced by the musculotropic stimulant, barium chloride, so effectively in comparison with atropine, papaverine is regarded as a musculotropic antispasmodic. However, after the muscle has been tensed by the neurotropic stimulant, acetylcholine, relaxation is brought about by an amount of atropine which is very small in comparison with the dose of papaverine needed to produce the same effect—atropine is a neurotropic antispasmodic.

TABLE I

Musculatropic and Neurotropic Activity of Papaverine and Atropine

	Maximum Effective Dilution		
	After Barium Chloride	After Acetylcholine	Reference
Papaverine	1 : 100,000	1 : 100,000	(3)
Atropine	1 : 2,500	1 : 5,000,000 to 1 : 50,000,000	(3)

	Minimum Effective Dose		
	After Barium Chloride	After Acetylcholine	Reference
Papaverine	0.8 mg.	0.5 to 0.8 mg.	(4)
Atropine	25 mg.	0.02 to 0.03 mg.	(4)

If the maximum effective dilution or the minimum effective dose of papaverine or atropine is expressed as unity, then the relative potency of a substance can be stated in terms of papaverine or atropine activity.

When a large number of compounds is to be tested, it is more practical in the preliminary tests to use the normal instead of the spastic intestine. Furthermore, it is considered to be of value to be able to compare the dose required for relaxation of the unstimulated with that necessary to relax the stimulated muscle.

Although the same general procedure is employed in the evaluation of antispasmodics on the isolated intestine, certain variations often are introduced and this circumstance, together with the lack of uniformity in the manner in which activity is expressed, makes impossible any reliable correlation of data published from different laboratories. Since there are certain to be differences in the sensitivity of the segments of the jejunum obtained from different rabbits, or even from the same animal, average values are far more reliable than those which represent a single experiment (5). An increase in the pH of the bath in which the intestinal strip is immersed produces a decided increase in the tonus, but a decrease in pH causes a fall in tonus; strips of uterus and ureters also respond to a change in pH in the same manner as the intestinal muscle (6). Whether or not this important factor is taken into account routinely is not apparent from the literature. Although it is customary to employ a strip of rabbit jejunum in the Magnus procedure, guinea pig intestine has occasionally been used. The bath which surrounds the strip may be Tyrode's, Locke-Ringer's, Locke's, or Broom & Clark's solution (7).

Barium chloride and acetylcholine are stimulants most commonly employed, but histamine (8, 9) has been utilized in place of the former, and carbamylcholine (Lentin) (10, 11), eserine salicylate (12), acetyl-β-methylcholine (13), or acetylcholine bromide (8, 14) instead of the latter. It was stated by Lehmann & Knoefel (14) that histamine is more sensitive to concentration differences, and is more reliable than barium chloride, and that atropine is more active against histamine than against barium chloride although papaverine is about equally effective against both spasmogenic agents. Carbamylcholine was selected by Wagner-Jauregg, Arnold & Born (10) because it is hydrolyzed less rapidly than acetylcholine.

Antispasmodic activity has been reported in terms of gram dosage (4), maximum effective dilution (3, 15), papaverine activity (16, 17), therapeutic index (18), and reciprocal activity (8, 9, 14). Instead of the effect produced on the isolated tissue (15, 17), only data obtained by observations on the intact animal may be published (18).

Finally, quite erroneous conclusions can be drawn with respect to

relative activity unless cognizance is taken of the inherent and unavoidable errors in pharmacological testing procedures. For example, in the case of an antispasmodic, a tabulated maximum effective dilution of 1 : 200,000 for compound A should be interpreted as a statement that this dilution is to be found probably somewhere between 1 : 100,000 and 1 : 300,000 and that, in the same table, a reported maximum effective dilution of 1 : 400,000 for compound B does not necessarily imply that the latter is exactly twice as active as compound A.

In view of this diversity in testing procedures and in the manner in which data are recorded, relative activity can be stated only in very general terms and consequently, it must be admitted, in a decidedly unsatisfactory manner.

Although we are not concerned in this survey of antispasmodics with any activity except that exhibited by a substance on the isolated intestine, it would be unfortunate if the impression were created that high effectiveness of a compound in this one respect is sufficient evidence of its therapeutic usefulness.

After one or more compounds have been selected from a group because of their satisfactory activity on the isolated intestine, then their effect in the intact animal must be observed. The technique required at this stage is much more difficult and time-consuming and, usually, it is gastrointestinal motility which is measured rather than the degree of relaxation produced in the spastic intestine. Furthermore, since smooth muscles in different parts of the body do not respond uniformly to the same drug, tests must be carried out with various isolated tissues, and on the organs *in situ,* in order that the influence of the product on different parts of the gastrointestinal tract and on the bronchi, gall and urinary bladders, ureter, and uterus may be observed. Any substance which seems to be active enough for clinical trials must be studied first for acute and chronic toxicity, and for its possible effects on respiration and blood pressure.

It may be stated at this point that our present-day antispasmodics are most effective on the gastrointestinal tract, and the probability that a single substance will be discovered which will be equally effective on all smooth muscles seems remote.

A series of esters has been tested, the members of which may be regarded as atropine in which the tropyl radical has been replaced by a variety of basic alcoholic groups. In other words, the compounds are basic esters of tropic acid, $ROOCCH(C_6H_5)CH_2OH$. R repre-

sents the following groups: β-dimethylaminoethyl (19), β-diethylaminoethyl (8), β-piperidinoethyl (18), γ-piperidinopropyl (18), β,β-dimethyl-γ-diethylaminopropyl (3, 8), β,β-dimethyl-γ-diethylaminopropyl (methobromide) (8), homotropyl (18), a basic β-substituted ethyl (18) and a basic γ-substituted propyl radical (18) in which the basic substituent is a condensed system composed of the cyclopentane and the 2-methylpyrrolidine rings. The evidence which has been obtained demonstrates without question that certain esters of tropic acid, in which the alcoholic radicals are much less complex than the one found in atropine, are effective antispasmodics. The primary acid phosphate of β,β-dimethyl-γ-diethylaminopropyl tropate, $(C_2H_5)_2NCH_2C(CH_3)_2CH_2OOCCH(C_6H_5)CH_2OH \cdot H_3PO_4$, is sold under the name of syntropan. Some of the basic esters of tropic acid mentioned above were synthesized initially by v. Braun, Braunsdorf & Räth (20), who stated that they are local anesthetics and mydriatics.

The β-piperidinoethyl (navigan) (21), β,β-dimethyl-γ-diethylaminopropyl (3, 4), and the β,β-dimethyl-γ-piperidinopropyl (3) esters of acetyltropic acid are antispasmodics. According to Fromherz (3), the second-mentioned ester is equivalent pharmacologically to the corresponding ester of tropic acid. The β,β-dimethyl-γ-diethylaminopropyl ester of O-methyltropic acid (3) is less active than that of tropic acid.

Esters which represent an atropine molecule in which only the acyl portion has been modified, and the tropyl radical left intact, have not been studied to any extent. The spasmolytic properties of ψ-tropyl benzilate have been demonstrated by Kreitmair (22); when tested on the cornea, the ester produced mydriasis and local anesthesia. The two latter effects are shown quite frequently by antispasmodics. The ester was studied also by Hesse, Niedenzu & Zeppmeisel (18) and by Halpern (4). According to Hesse *et al.* it is more toxic than atropine, and its effect on the intestine is no greater than that of other synthetic antispasmodics. A clinical report was published by Kroner (23).

The type of ester in which both the alcoholic and the acyl radical are different from those in atropine is the one which has been investigated most extensively. Hundreds of such esters have been tested pharmacologically in connection with antispasmodic investigations. A large number of them may be described as basic ethyl or propyl esters of disubstituted acetic acids, and, at the present time, it is especially among these compounds that useful antispasmodics are sought. It was quite logical that the esters to receive special attention should

be those of disubstituted acetic acids since it was evident that tropic acid, if it is regarded as phenyl-(hydroxymethyl)-acetic acid rather than as α-phenyl-β-hydroxypropionic acid, belongs to this class of acids. Furthermore, since it was more difficult to synthesize an acid which contained a hydroxymethyl group than one in which the substituent was merely methyl, and since there was no *a priori* evidence that the hydroxyl group would confer any desirable properties on the molecule, it followed quite naturally that, sooner or later, basic-alkyl esters not only of phenylmethylacetic but also of a variety of phenylalkylacetic acids would be synthesized.

It was Halpern (4) especially who called attention to the potency of esters of phenylalkylacetic acids. His investigation, which covered more than sixty compounds, was not limited to the aromatic-aliphatic class but included also esters of aliphatic and aromatic acids. The various acids were esterified with such basic alcohols as β-diethylaminoethyl, γ-diethylaminopropyl, β,β-dimethyl-γ-diethylaminopropyl, and α-methyl-δ-diethylaminobutyl alcohol. The esters were tested in the form of their hydrochlorides, a few as methoiodides. The actual doses which produced relaxation in the isolated intestine, stimulated with barium chloride or acetylcholine, were reported for only about one third of the compounds while the effects observed for the others were stated in the form of very general conclusions. He reported that the basic alcohol employed in the preparation of the ester establishes the quality (musculotropic or neurotropic) of the activity and the acid the potency, and that esters derived from basic ethanols or propanols are mainly neurotropic, while those which contain basic pentyl radicals are principally musculotropic antispasmodics. Although such generalizations may be warranted as far as his products are concerned, it should not be assumed that they apply universally until mass data have been examined. Halpern was impressed very favorably by the behavior of β-diethylaminoethyl phenylpropylacetate (β-diethylaminoethyl ester of α-phenylvaleric acid); consequently this substance was studied in the intact animal (4, 24). In an attempt to find some explanation for the activity of this ester, he tested β-diethylaminoethanol, and phenylpropylacetic acid in the form of its sodium salt; neither compound produced the slightest relaxation in the intestine stimulated by barium chloride.

The earliest pharmacological publication which deals with antispasmodics, especially in such a manner that the influence of the acyl radical in esters could be seen, is that of Fromherz (3). He tested the

β,β-dimethyl-γ-diethylaminopropyl esters of the following acids: tropic, acetyltropic, acetylmandelic, acetylatrolactinic, benzilic, phenyl-β-bromoallylacetic, phenylbenzylacetic, cyclohexylallylacetic, α-benzyl-β-acetoxybutyric, and levulinic acid. Probably the reason β,β-dimethyl-γ-diethylaminopropyl alcohol was chosen for the preparation of a series of esters was that this alcohol was used for the manufacture of the very active local anesthetic, larocain, and because the alcohol could be obtained readily by the Mannich reaction (25) after isobutyraldehyde had become available commercially. The esters were tested as mineral acid salts and as quaternary compounds. The benzilic acid ester was found to be very active but was considered to be too toxic for practical use. The most satisfactory product seemed to be the ester of *dl*-tropic acid (syntropan) (3, 26 to 29). Many esters of the syntropan type have been described in the patent literature (30, 31).

In 1936, three years after the first account of syntropan had appeared in the medical literature, an article was published (32) which described the favorable properties of another ester antispasmodic called trasentin. This substance is β-diethylaminoethyl diphenylacetate hydrochloride, $(C_6H_5)_2CHCOOCH_2CH_2N(C_2H_5)_2 \cdot HCl$. In this compound the alcoholic radical, and from some standpoints also the acyl group, is simpler than that found in syntropan. Since the pharmacological and clinical papers which deal with the respective merits of syntropan and trasentin (12, 33) are so numerous, and because there is not complete accord in the experimental data, no brief, adequate discussion of these esters can be given in this review.

More recently the properties of a hexahydro derivative of trasentin, called trasentin-6H (trasentin-H) (5, 12) have been reported. This ester is β-diethylaminoethyl phenylcyclohexylacetate hydrochloride, $(C_6H_5)(C_6H_{11})CHCOOCH_2CH_2N(C_2H_5)_2 \cdot HCl$. Important chemical publications which deal with the preparation of trasentin and trasentin-6H are those of Miescher & Hoffmann (34, 35, 36).

In connection with the study of trasentin-6H, a detailed pharmacological report of a number of products was published by Meier & Hoffmann (5). They described the behavior of twenty-three esters and amides of the following mono-, di-, and trisubstituted acetic acids: phenyl-, cyclohexyl-, phenylpropyl-, cyclohexylpropyl-, diphenyl-, tetrahydrodiphenyl-, phenylcyclohexyl-, dodekahydrodiphenyl-, and triphenylacetic acid. At least ten different basic alcohols were used in the preparation of the products. The amides studied were those which corresponded to the esters; for example, the β-diethylaminoethylamide

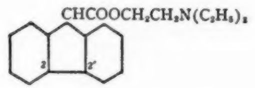
of diphenylacetic acid and β-diethylaminoethyl diphenylacetate. The esters and amides were tested as salts such as the hydrochloride and as quaternary compounds, usually methochlorides or methobromides. In the case of β-diethylaminoethyl esters, those obtained from the disubstituted acetic acids were found to be more active than those prepared from mono- or trisubstituted acetic acids. The esters of diphenylacetic acid are active musculotropic as well as neurotropic spasmolytics, but are exceeded in potency by esters of partially hydrogenated diphenylacetic acids. The latter are more effective than β-diethylaminoethyl phenylpropylacetate which was examined by Halpern. The introduction of nuclear alkoxy groups into esters of diphenylacetic acid did not increase activity. Esters which contained an α-hydroxyl group exhibited the side-effects of atropine to a greater degree than hydroxyl-free esters. In comparisons of β-diethylaminoethyl esters with other basic-alkyl esters it was found that the latter possess no advantages over the former with respect to activity or toxicity. The amides behave qualitatively like the esters. However, in the case of the amides, those of mono- and trisubstituted acetic acids are more active than those of corresponding disubstituted acids. Thus the sulfate of triphenylacetic β-diethylaminoethylamide is almost as active as the hydrochloride of β-diethylaminoethyl diphenylacetate. In general, it was found that when an ester was converted into a quaternary compound, such as the methochloride, toxicity and neurotropic activity increased while musculotropic activity decreased. Somewhat comparable effects were noticed by Wagner-Jauregg, Arnold & Born (10) in the case of quaternary derivatives of esters of diaralkylacetic acids. Without question, β-diethylaminoethyl phenylcyclohexylacetate (trasentin-H), that is, the ester of hexahydrodiphenylacetic acid, seems to be the most satisfactory compound. Its neurotropic activity on the intestine almost equals that of atropine, its musculotropic activity is greater than that of papaverine, and it relaxes the uterus.

A comparative study was made of trasentin and trasentin-6H by Graham & Lazarus (12). They found that the latter is the more active both on the isolated and on the intact intestine. Its neurogenic activity is comparable to that of atropine, and its myogenic activity to that of papaverine. Trasentin-6H, according to them, is 18 per cent more toxic than trasentin and about 25 per cent more toxic than atropine. However, this does not militate against its use in view of its very high activity and its relative freedom from the side-effects on the pupil, heart, and sweat glands which are characteristic of atropine.

A few nuclear substituted alkyl and basic-alkyl esters of phenylcyclohexylacetic acid were described by Hoffmann (37). Methyl *p*-aminophenylcyclohexylacetate was found to be a weak antispasmodic, and methyl *p*-(β-diethylaminoethylamino)-phenylcyclohexylacetate was said to be less active than trasentin-H. In the case of β-diethylaminoethyl *p*-aminophenylcyclohexylacetate, the neurotropic activity was stated to be slightly higher than that of trasentin-H, and the musculotropic activity somewhat less; it is especially interesting to note that this *p*-amino compound, in contrast to trasentin-H, proved to be inactive as a local anesthetic in 1 per cent solution.

Recently Gilman *et al.* (38) tested a number of basic esters of diphenylacetic acid for local anesthetic activity, and found that all of them not only are local anesthetics but are characterized by antispasmodic and parasympatholytic properties. Especially interesting is their statement that trasentin compares favorably with many local anesthetics in clinical use; it is more active than cocaine both as a block and surface anesthetic, and is less toxic. They also contended that β-dibutylaminoethyl diphenylacetate hydrochloride undergoes spontaneous hydrolysis in solution.

During this year a third ester, β-diethylaminoethyl fluorene-9-carboxylate hydrochloride, appeared on the market under the name pavatrine. The structural relationship between this compound and trasentin is a close one. If a bridge were established in the latter between carbon atom 2 of one benzene ring and carbon atom 2′ of the other benzene ring, the new structure would represent pavatrine, IV(*a*).



IV(*a*), (*b*) CH_2, (*c*) O, (*d*) S, (*e*) NH

The series of products which was prepared during the search which led to the discovery of pavatrine was described recently by Burtner & Cusic (8). Although quite a variety of esters was studied, special emphasis was placed on the synthesis of fluorene compounds because during earlier research on local anesthetics these investigators had found that cyclization of certain derivatives of polynuclear carboxylic acids enhanced activity. According to the published data (8, 14), the introduction of a double bond between two nuclear carbon atoms in

basic esters of diphenylacetic acid in many cases increased antispasmodic activity with little or no increase in toxicity.

In their second communication (9) Burtner & Cusic described basic esters of other bridged forms of diphenylacetic acid. In these compounds the 2,2′ carbon atoms were joined, not by a single bond, as in formula IV(*a*), but through CH_2, O, S, or NH, IV(*b*) to (*e*). One of the effects of a CH_2 bridge is illustrated in the case of β-diethylaminoethyl 9,10-dihydroanthracene-9-carboxylic acid, IV(*b*). This substance was found to be twenty times as active as the corresponding ester of fluorene-9-carboxylic acid against spasms induced by histamine (9); however, if the barium chloride value (14), instead of that obtained with histamine, is accepted as a measure of musculotropic activity it will be seen that the ester is no more active than that of the fluorenecarboxylic acid. The dihydroanthracene, IV(*b*), proved to be less effective than the fluorene derivative against acetylcholine. In β-diethylaminoethyl xanthene-9-carboxylate, IV(*c*), both the musculotropic and the neurotropic effects are intensified about two fold while there is no appreciable change in toxicity. The thioxanthene ester, IV(*d*), is less active. The ester which contains a dihydroacridine nucleus, IV(*e*), that is an NH bridge, proved to be less effective against both types of spasm and is more toxic.

A more detailed pharmacological report which deals with many of the esters prepared by Burtner & Cusic was published by Lehmann & Knoefel (14). The relative spasmolytic activities of forty-five products were determined; among them were papaverine, atropine, syntropan, trasentin, pavatrine, seven basic esters of diphenylacetic acid, and nine basic esters of fluorene-9-carboxylic acid. It was shown that many of the esters, including pavatrine, are local anesthetics. The latter produces mydriasis when applied to the cornea. In general, the most effective esters are those in which the basic-alkyl group is β-diethylaminoethyl.

Esters of 9-hydroxyfluorene-9-carboxylic acid, among them the tropyl, ψ-tropyl, and the β-diethylaminoethyl esters, have been described by Wolfes & Hromatka (39) as antispasmodics, and basic-alkyl esters of fluorenonecarboxylic acids have been discovered by Ray & Rieveschl (40) which are antispasmodics and local anesthetics.

In view of the high activity of β-diethylaminoethyl diphenylacetate, it was of interest to study esters of xenylacetic acid, that is, esters in which two phenyl groups are attached to each other as in biphenyl (xenyl). The preparation of basic esters of the general formula

p-$C_6H_5C_6H_4RCHCOOR' \cdot HCl$, in which R represents H, C_6H_5, C_6H_{11}, CH_3, C_2H_5, or C_3H_7, and R′ is a basic radical, has been described by Blicke & Grier (41). It was shown by Lewis, Lands & Geiter (42) that β-piperidinoethyl methyl-*p*-xenylacetate hydrochloride produces relaxation in the isolated intestine in a dilution of from 1 : 2,000,000 to 1 : 4,000,000.

Esters of acetic acid into which two phenyl groups are introduced in the form of a condensed system such as α-naphthyl have been prepared by Blicke & Feldkamp (43). These esters conform to the general formula α-$C_{10}H_7RCHCOOR'$; R represents H, CH_3, C_2H_5, or C_6H_5, and R′ a basic radical. Highly active compounds have been found in this series.

Cheney & Bywater (17) described about thirty-five esters and amides which were characterized by the presence of a morpholinoalkyl group such as β-morpholinoethyl, γ-morpholinopropyl, β-methyl-β-morpholinopropyl, or β,β-dimethyl-γ-morpholinopropyl. A considerable variety of acids was used for the preparation of the esters, and among the acids were diphenylacetic and phenylcyclohexylacetic acid. The papaverine activities were reported but a more detailed description of their pharmacological properties has been published by Rowe (15), and by Yonkman *et al.* (44). Tests were made on the unmedicated intestinal strip with papaverine as a standard. Many of the esters were found to be less active than the alkaloid, a few equaled it, and several exceeded it in potency. Based on their research, the following conclusions were reached. The acid employed for the preparation of an active ester should be a disubstituted acetic acid in which at least one group is aryl, since esters of monoaryl, di- and trialkyl, and monoalicyclic acetic acids are relatively inactive. In one instance, the activity was increased only slightly by the substitution of cyclohexyl for one phenyl radical in an ester of diphenylacetic acid; in another case, the activity was decreased when this substitution was made. This is interesting in view of the fact that trasentin-6H is decidedly more effective than trasentin. The amides, for example the β-morpholinoethylamide of diphenylacetic acid, were shown to be less active than the corresponding esters.

Wagner-Jauregg, Arnold & Born (10) prepared a series of β-diethylaminoethyl esters of disubstituted acetic acids of the following general formula: $(RCH_2)(R')CHCOOCH_2CH_2N(C_2H_5)_2$; R = aryl or aralkyl; R′ = alkyl, cycloalkylalkyl, aryl, or aralkyl. The most active products proved to be those which contained a benzyl group. The

ester of dibenzylacetic acid was found to be three to four times as potent as papaverine. The β-diethylaminoethyl ester of di-(β-phenylethyl)-acetic acid was said to be three times as active as papaverine but the corresponding ester of di-(β-cyclohexylethyl)-acetic acid proved to be only one twentieth as effective as the alkaloid. They showed that in some instances the papaverine activity of the ester hydrochloride was increased greatly by conversion of the ester into a quaternary derivative which was effected through addition of such compounds as ethyl bromide, octyl bromide, or benzyl bromide to the ester. On the whole, the atropine activity for their products was low. The compounds which represented the most favorable combination of both musculotropic and neurotropic properties were found to be β-diethylaminoethyl phenylbenzylacetate and β-diethylaminoethyl isopropylbenzylacetate. β-Diethylaminoethyl dibenzylacetate hydrochloride was administered to human subjects but since it produced vomiting and a feeling of faintness, the product was considered unfit for clinical use. It was shown that two esters, β-diethylaminoethyl di-(β-phenylethyl)-acetate and tropyl dibenzylacetate, in the form of their hydrochlorides produced local anesthesia when applied to the rabbit's cornea.

Basic esters of benzilic acid, diphenylhydroxyacetic acid, deserve special mention because they have been examined as potential antispasmodics in so many instances (3, 4, 8, 14, 17, 22, 23, 45). Although a number of them are quite active, the general opinion seems to be that they are too toxic for therapeutic use. In at least some of them, local anesthetic (14, 38) and mydriatic (46) properties are developed to a high degree. Gilman *et al.* stated that β-diethylaminoethyl benzilate compares favorably with papaverine and approaches atropine in parasympatholytic activity.

Quaternary compounds, obtained from basic esters by interaction with an alkyl or aralkyl halide, often are highly active spasmolytics; however, they are apt to be more toxic than the tertiary amino compound from which they were prepared. Whenever esters are synthesized in order that they may be examined for antispasmodic or mydriatic activity, it is customary to convert a few of them into quaternary products.

Eumydrin, methylatropinium nitrate, was introduced as a mydriatic about 1903 (47). Because of its more recent application as a spasmolytic, especially in the treatment of pyloric stenosis of infants, and because of the conflicting statements in the literature, it was studied by Graham & Lazarus (48). They found that its potency on the in-

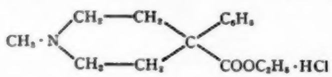
testinal tract is equal to that of atropine but it is about three times as toxic.

Novatropine, methylhomatropinium bromide, and atropine, according to Quigley (49), produce about the same degrees of inhibition on the human stomach, and on the stomach and colon of unanesthetized dogs.

During the last few years several basic esters of unusual interest have been studied. The novel structural feature of these products is the presence of the basic nitrogen in the acyl instead of in the alcoholic radical. The pharmacology of one of these esters, designated as benzyl β-dimethylamino-α-phenyl-α-ethyl-propionate hydrochloride, was described by Unna (50). It would be just as proper to regard this ester as a trisubstituted acetate, benzyl dimethylaminomethylphenylethylacetate, $(CH_3)_2NCH_2(C_6H_5)(C_2H_5)CCOOCH_2C_6H_5$, and thus, to some extent, correlate it with the basic esters of disubstituted acetic acids which have been discussed. The ester has good papaverine activity but is a weak neurotropic antispasmodic. It produces anesthesia on rabbit cornea, and also by intradermal injection.

Ethyl diphenyl-α-(β-morpholinoethyl)-acetate has been mentioned in the patent literature (51) as an antispasmodic.

Another ester, one which has attracted a great amount of attention, is called demerol or dolantin, V. It is the hydrochloride of ethyl 1-methyl-4-phenylpiperidine-4-carboxylate (52, 53). In 1939, it was announced by Eisleb & Schaumann (54) that the ester exhibits



V

not only the antispasmodic activity of both atropine and papaverine, but also an analgesic effect comparable to that of morphine. Naturally, in view of such statements, especially the one relative to analgesic properties, the compound would be examined extensively by pharmacologists (55 to 60) and clinicians (61, 62). Gruber, Hart & Gruber, Jr. (63) found demerol to be less effective than papaverine hydrochloride on isolated tissues. However, the chief interest in demerol lies not so much in its possible usefulness as an antispasmodic but in its value as a morphine substitute. Probably some time will elapse before definite and final conclusions are reached with regard to its analgesic potency and its freedom from addiction.

Since demerol is the first, relatively simple synthetic product discovered which possesses a structure radically different from morphine yet seems to resemble the latter in activity, it is especially interesting to know how the substance was discovered in the I. G. Farbenindustrie laboratories. This was disclosed by Schaumann (11) who stated that a clue was obtained by observation of the peculiar position of the tails of mice during toxicity tests. Presumably he referred to the S-shape of the tail, commonly known as the Straub reaction. He stated that, even though this effect is not necessarily characteristic of morphine, its observance after the administration of a compound could be taken as an indication that the substance might have some property in common with the alkaloid. In this same article, Schaumann presented data with regard to the relative antispasmodic and analgesic potencies of a large number of products related to demerol in structure.

In addition to the basic esters just discussed, interesting as well as clinically important antispasmodics are to be found in another group of compounds which cannot be characterized any more definitely than by the term amines.

Papaverine, 1-(3′,4′-dimethoxybenzyl)-6,7-dimethoxyisoquinoline, had been isolated in 1848 but its possible therapeutic importance was not recognized until the discovery by Pal (64, 65, 66), in 1913, that the alkaloid possesses antispasmodic properties. This substance became of increasing interest, and by 1928 some concern was felt about the amount which would be available to meet clinical demands (67). Papaverine is isolated incidental to the extraction of morphine from opium, but after the quantity of morphine to be placed on the market had been fixed by international agreement, the amount of papaverine which could be obtained legally from this source also was limited. Due to the high cost of the raw material and the low yields of the product, manufacturing processes, which might have been used fifteen years ago, and by means of which the supply of papaverine could have been augmented, did not seem feasible. Consequently, attention was directed to synthetic products, very similar to papaverine in structure, which had been described in the literature, and a number of new, papaverine-like compounds were synthesized. In these new compounds two methylenedioxy groups, for example, were substituted for the four methoxy groups in papaverine, or the latter were partially replaced by hydrogen, a methyl group was attached to the 3 carbon atom, the benzyl group was exchanged for phenyl, β-phenylethyl, di-

phenylmethyl, etc. Some of these substances were discovered to be equal or superior to the alkaloid in potency and in their therapeutic index, and could be manufactured at a more reasonable cost. Attention was directed especially to eupaverin (67 to 70) and perparin (68, 69); the former is 1-(3′,4′-methylenedioxybenzyl)-3-methyl-6,7-methylenedioxylisoquinoline hydrochloride, the latter 1-(3′,4′-diethoxybenzyl)-6,7-diethoxyisoquinoline hydrochloride. It seems probable that these substances, due to the great similarity in structure, possess at least some of the defects of papaverine. Parenteral administration of papaverine in the form of its salts, such as the hydrochloride, is unsatisfactory for the following reasons: due to the relatively low water-solubility, only solutions of very limited concentration can be prepared; because of the weakly basic nitrogen in the molecule, the salts hydrolyze, hence the solutions are acidic (68), and cause pain when injected; and, the drug is not absorbed satisfactorily since the insoluble papaverine base is precipitated by the hydrolysis of the salt (16). The spasmolytic activity of the 1-methyl, 1-phenyl, 1-benzyl, and 1-phenylethyl derivatives of 3-methyl-3,4-dihydroisoquinoline and of 3-methyl-1,2,3,4-tetrahydroisoquinoline was determined by Cunningham & Fellows (71).

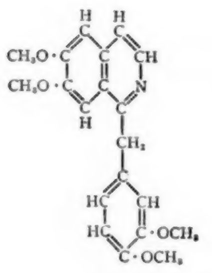
Macht (72, 73) attributed the spasmolytic properties of papaverine to the presence of the benzyl group. Because of Macht's affirmation, simple esters which contained the benzyl group, such as benzyl succinate, benzyl fumarate, and benzyl benzoate, appeared on the market some years ago. Although these compounds may be effective for certain conditions, their endorsement as strong antispasmodics does not seem to be warranted on the basis of pharmacological evaluations.

Substituted hydroxybenzyl alcohols have been described by Dunning, Jr., Dunning & Reid (74), and were tested with saligenin (*o*-hydroxybenzyl alcohol) as a standard. The latter, to a limited degree, is an antispasmodic, antiseptic, and local anesthetic.

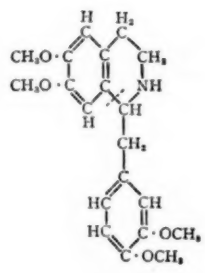
In order to obtain information relative to the significance of the benzyl group in papaverine, Kreitmair (75) studied the properties of the hydrochlorides of the following four compounds: (*a*) 1-benzyl-3-methyl-6,7-methylenedioxyisoquinoline; (*b*) 1-phenyl-3-methyl-6,7-methylenedioxyisoquinoline; (*c*) 1-(3′,4′-methylenedioxybenzyl)-3-methyl-6,7-methylenedioxyisoquinoline; and (*d*) 1-(3′,4′-methylenedioxyphenyl)-3-methyl-6,7-methylenedioxyisoquinoline. It is apparent that compounds (*a*) and (*b*) are alike except that the former contains a benzyl group, the latter a phenyl; the same statement can be

made for compounds (*c*) and (*d*). The benzyl compounds were found to have about the same order of activity as papaverine but the phenyl compounds are ten times as active on the isolated intestine. From this, and other investigations (76, 77), it is evident that the benzyl radical in papaverine plays a subordinate role as far as antispasmodic activity is concerned. Other attempts to find active analogues of papaverine have been described in the literature (67, 78, 79, 80).

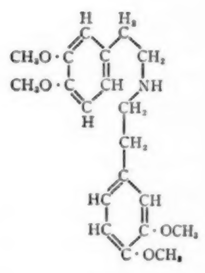
In 1939, Buth, Külz & Rosenmund (16) published their first chemical article which describes the search, begun ten years earlier, for papaverine substitutes. Since it seemed that isoquinoline compounds, in general, probably would not be free from the defects of papaverine (VI), they decided to synthesize derivatives of a new parent compound, namely di-(β-phenylethyl)-amine. It was consideration of the following facts which led them to adopt this structural pattern. In certain instances, a completely saturated ring may be opened without loss of activity. 1,2,3,4-Tetrahydropapaverine (VII) is a weak antispasmodic. If one of the rings in this compound were opened at the point shown by the dotted line in formula VII, and two



VI



VII



VIII

hydrogen atoms added, di-(β-3,4-dimethoxyphenylethyl)-amine (VIII) would be obtained. Since this amine with its four methoxy groups is more difficult to synthesize than di-(β-phenylethyl)-amine, a sample of the latter was prepared first in order that it might be tested for antispasmodic activity. It was found that this substance does relax smooth muscle to some degree, and it was but natural to expect that derivatives could be found which would exhibit enhanced activity. This proved to be the case. The number and variety of such deriva-

tives which can be obtained by relatively simple processes is surprisingly large. About thirty of them were described in the publication by Buth *et al.* Most of the amines conform to the following general formula: Aryl–CH_2–CH(R)–NH–CH(R′)–CH_2–Aryl, where Aryl = phenyl, methoxyphenyl, dimethoxyphenyl, or methylenedioxyphenyl; and R and R′ = hydrogen, methyl, ethyl, propyl, isobutyl, benzyl, or phenyl. Some of the amines proved to be less active, others more active than papaverine. It was found that, whereas introduction of methoxy or methylenedioxy groups into the benzene nucleus does not increase activity, compounds more potent than papaverine were obtained by replacement of a *beta* hydrogen of the carbon chain by methoxy, alkyl, benzyl, or phenyl. Some of the amines also produced local anesthesia and others, like lobeline, were found to be respiratory stimulants.

In another investigation, Külz & Rosenmund (81) studied unsymmetrical amines of the following general type: $C_6H_5CH_2CH(R)NH$-$CH(R')CH_2C_6H_5$, where R = H or CH_3, and R′ = C_2H_5, C_3H_7, C_4H_9, or C_6H_5. They found that activity increased with an increase in the carbon content of R′. Since salts of these amines are not soluble enough, and because the amines would be too costly to manufacture, another series of amines was prepared in which chains with an increasing number of carbon atoms were attached to the nitrogen atom in di-β-phenylethylamine: $(C_6H_5CH_2CH_2)_2N-R$, where R = CH_3 C_2H_5, C_3H_7, or C_6H_{13}. The hexyl compound is one to two times as effective as papaverine. However, it was found that more potent products were obtained when an alkyl radical was attached to the benzene ring. The papaverine activity for β-phenylethyl-β-*p*-tolylethylamine was found to be 1.5 and that for di-(β-*p*-tolylethyl)-amine was 2. Finally, since salts of the amines which contain an *N*-alkyl group are more water-soluble, a group of *N*-alkyl amines was synthesized in which the carbon chain between the phenyl group and the nitrogen atom was lengthened. A compound of this type, ethyldi-(γ-phenylpropyl)-amine hydrochloride, $(C_6H_5CH_2CH_2CH_2)_2NC_2H_5 \cdot$-HCl, was placed on the market under the name sestron (82, 83). It has a papaverine activity of 2.3. After all of the requirements for a clinically useful product had been taken into consideration, this amine was considered to be the most satisfactory member of the group of 140 compounds which had been studied.

Primary, secondary, and tertiary amines which contain at least one β-phenylethyl group were prepared by Kindler & Peschke (84). They

found that compounds which contain a nuclear ethoxy group are more effective than corresponding methoxy derivatives.

Since one of the rings of the isoquinoline nucleus in papaverine can be completely hydrogenated and opened without complete loss of antispasmodic activity, what would be the effect if both rings were completely saturated and then one of them ruptured at the same point shown in formula VII? In order to answer this question, an extensive series of cyclohexyl amines of the general formula $C_6H_{11}(CH_2)_x$-N(H or alkyl)$(CH_2)_xC_6H_{11}$ were prepared by Blicke & Monroe (85), and Blicke & Zienty (86). Many of them in high dilution produced relaxation in the isolated intestine, and methyldi-(β-cyclohexylethyl)-amine hydrochloride was examined pharmacologically more in detail (87, 88).

After the discovery of the activity of cycloalkylalkylamines it was only logical to expect that spasmolytics might be found among the amines which are purely acyclic in structure. In fact, as early as 1933, active unsaturated, aliphatic amines had been found. Mügge (89) reported that a number of heptenes and octenes had been sent to him by the Knoll firm. Upon investigation, he found that methylaminoisooctene, or more specifically 2-methyl-6-methylaminoheptene-2, $(CH_3)_2C = CHCH_2CH_2CH(NHCH_3)CH_3$, is an antispasmodic. This substance, in the form of an aqueous solution of the very hygroscopic hydrochloride or the solid mucate, appeared on the market under the name octin (octinum) (90). A number of unsaturated, aliphatic amines were studied by Hesse, Niedenzu, & Zeppmeisel (18), and according to them 2-aminohexene-5, $CH_2 = CHCH_2CH_2CH(NH_2)$-$CH_3$, is more active than octin.

During the continuation of the study of synthetic antispasmodics under the writer's supervision, a number of simple, saturated, aliphatic amines were synthesized (91). It was found that some of these compounds, among them hexyloctyl-, dibutyloctyl-, and ethyldiheptylamine, did exhibit spasmolytic activity. Although a total of 433 amines were prepared (85, 86, 92, 93) and about 60 per cent of them were found to produce relaxation, at least to some degree, in the isolated intestine, no very definite relationship between structure and antispasmodic activity was apparent. A number of saturated aliphatic (94, 95) and unsaturated aliphatic amines (96, 97), stated to possess spasmolytic properties, have been mentioned in the patent literature.

In a few instances, comparisons have been made between the in-

tensity of the spasmolytic activity of a given substance and the magnitude of a certain physical property. Halpern (4) was unable to establish any direct relationship between surface tension and antispasmodic potency. A few years earlier, v. Issekutz, Leinzinger & v. Issekutz, Jr. (98) had studied a number of miscellaneous types of products—alkaloids, compounds of the papaverine type, local anesthetics, alcohols, urethanes, substituted ureas, etc.—with respect to their action on the isolated intestine, and determined also their surface tension. They claimed that a direct relationship exists between this physical property and the effect on the muscle. Junkmann (99) measured the surface tension of a number of compounds and stated that to some extent there seemed to be a parallel relationship between surface tension and spasmolytic activity.

Fromherz (3) was the first to investigate the relative antispasmodic potency of optically active isomers. He found that after stimulation of the isolated intestinal strip with acetylcholine, *levo*-β,β-dimethyl-γ-diethylaminopropyl acetyltropate was ten to twenty times as effective as the *dextro* isomer, but after barium chloride stimulation the difference between the activity of the two isomers was not nearly as marked. The *levo* isomer produced mydriasis when applied to cat cornea in 0.5 per cent solution whereas no effect was observed with a 5 per cent solution of the *dextro* compound. Buth, Külz & Rosenmund (16) found that the *racemic* and optically active forms of di-(β-phenylisopropyl)-amine exhibit about the same degree of spasmolytic activity; the *meso* form is somewhat less effective, and is three times as toxic as the *racemic* modification. In the relaxation of the gut after treatment with acetyl-β-methylcholine or barium chloride, *l*-cinnamylephedrine is only slightly more effective than *d*-cinnamylephedrine according to Schultz (13).

Although the basic esters and amines represent the classes of compounds which have been studied most thoroughly, antispasmodic activity is not found exclusively in these groups. Fromherz (100) tested hypnotics of the amide and ureide type, as well as a number of structurally related compounds, for antispasmodic activity. These substances do not seem to have the high potency of esters and amines; their effect appears to be a direct one on the muscle.

A long series of substituted acetamides was examined by Junkmann (99) for hypnotic and spasmolytic properties. The strongest antispasmodic activity was found in compounds which contained 12 to 17 carbon atoms and which were inactive as hypnotics. Tributyl-

acetamide was submitted for clinical trials. Since so many antispasmodics produce local anesthesia to some degree, it is interesting to note that Junkmann employed β-diethylaminoethyl *p*-aminobenzoate (procaine), an exceedingly weak antispasmodic, as a standard. Halpern (4) found α-methyl-δ-diethylaminobutyl *p*-aminobenzoate to be inactive as a spasmolytic.

Billman & Hidy (101) synthesized α-aminodiphenylacetamide and N,N′-substituted derivatives of the general formula $(C_6H_5)_2C$-(NHR)CON′HR in which R represents alkyl. These compounds were tested for antispasmodic and anticonvulsant properties. The most active antispasmodic was found to be α-aminodiphenylacetamide.

Certain hypnotics of the barbituric acid type depress excised smooth muscles (102 to 105). Epinephrine relaxes the musculature of the gastrointestinal tract but no practical use can be made of this effect since doses large enough to produce the desired response promote marked cardiovascular disturbances.

Cinnamylephedrine, $C_6H_5CH(OH)CH(CH_3)N(CH_3)CH_2CH=$-$CHC_6H_5$, was described as a local anesthetic by Ehrhart (106). Its action on smooth muscle was studied by Schultz (13, 107). He found that *l*-cinnamylephedrine hydrochloride is slightly more active than the *dextro* isomer, and about two times as active as papaverine, in relaxation of spasm produced by acetyl-β-methylcholine or barium chloride. Anesthesia was produced when cinnamylephedrine was applied to rabbit cornea but there was no change in the size of the pupil. 4-(1-Methyl-4-phenyl)-piperidyl methyl ketone and a number of analogous compounds have been patented as antispasmodics and anodynes (108). α-(N-Methylpiperidyl)-methylpropylcarbinol, $(CH_3NC_5H_9)(CH_3)$-$(C_3H_7)COH$, has a papaverine-like action on the muscles of the intestine (109). Antispasmodics have been found in the ω-aminoalkylquinoline series (110). α-Phenyl-β-hydroxy-β-(*o*-chlorophenyl)-ethylisoquinolinium bromide was stated by Kröhnke (111) to be twenty times as active as papaverine. Certain 4-aralkyl-3-keto-3,4-dihydro-1,4-benzoxazines are antispasmodics or analgesics (112), and the spasmolytic activity of a piperidinomethylbenzodioxane has been studied by Hazard & Moisset de Espanés (113). Taurine has been shown (114) to be an antispasmodic but its activity has been stated (18) to be less than that of papaverine.

As far as a relationship between structure and antispasmodic activity is concerned, the general statement can be made that this type of activity is not limited to one particular class of compounds, and that

within a given class of active compounds no one specific radical seems to be wholly responsible for the activity.

In view of all the attempts which have been made to correlate a physical property or a chemical structural unit with pharmacological activity, the writer wonders if it is not futile to endeavor to establish a broad generalization between any single physical property, or any one structural group, and activity. It would seem that it is not one particular physical or chemical property but the over-all properties of a molecule which play the important role, and these are influenced not only by the group which is supposed to represent the "active" part of the structure, but also by the residue or carrier of this group.

It is in the practical aspects of the field that decided progress has been made. Many compounds have been found which exhibit not only the musculotropic activity of papaverine but also the neurotropic activity of atropine and are free from the undesirable and unavoidable side-effects of the latter drug. Furthermore, because of their favorable therapeutic index, several active products have been discovered which are suitable for clinical use.

Our present-day antispasmodics are most effective against spasms of the gastrointestinal tract. Further research is needed in order to discover still more potent compounds and products which will relieve smooth muscle spasms of other organs.

In conclusion, attention should be called to a brief survey by Raymond (115) on recent developments in synthetic antispasmodics.

LITERATURE CITED

1. MAGNUS, R., *Arch. ges. Physiol. (Pflügers)*, **102**, 123–51 (1904)
2. MAGNUS, R., *Arch. ges. Physiol. (Pflügers)*, **103**, 515–40 (1904)
3. FROMHERZ, K., *Arch. exptl. Path. Pharmakol.*, **173**, 86–128 (1933)
4. HALPERN, B. N., *Arch. intern. pharmacodynamie*, **59**, 149–94 (1938)
5. MEIER, R., AND HOFFMANN, K., *Helv. Med. Acta*, **7**, Suppl. VI, 106–25 (1941)
6. GRUBER, C. M., *J. Pharmacol.*, **30**, 149–62 (1927)
7. BROOM, W. A., AND CLARK, A. J., *J. Pharmacol.*, **22**, 59–74 (1924)
8. BURTNER, R. R., AND CUSIC, J. W., *J. Am. Chem. Soc.*, **65**, 262–67 (1943)
9. BURTNER, R. R., AND CUSIC, J. W., *J. Am. Chem. Soc.*, **65**, 1582–85 (1943)
10. WAGNER-JAUREGG, T., ARNOLD, H., AND BORN, P., *Ber. deut. chem. Ges.*, **72**, 1551–61 (1939)
11. SCHAUMANN, O., *Arch. exptl. Path. Pharmakol.*, **196**, 109–36 (1940)
12. GRAHAM, J. D. P., AND LAZARUS, S., *J. Pharmacol.*, **69**, 331–41 (1940)
13. SCHULTZ, F. H., *J. Pharmacol.*, **70**, 283–92 (1940)
14. LEHMANN, G., AND KNOEFEL, P. K., *J. Pharmacol.*, **74**, 274–83 (1942)
15. ROWE, L. W., *J. Am. Pharm. Assoc.*, **31**, 57–59 (1942)
16. BUTH, W., KÜLZ, F., AND ROSENMUND, K. W., *Ber. deut. chem. Ges.*, **72**, 19–28 (1939)
17. CHENEY, L. C., AND BYWATER, W. G., *J. Am. Chem. Soc.*, **64**, 970–73 (1942)
18. HESSE, E., NIEDENZU, M., AND ZEPPMEISEL, L., *Klin. Wochschr.*, **15**, 1164–66 (1936)
19. GERMAN PATENT 382,137, *Chem. Zentr.*, **95**, Part I, 1105 (1924)
20. BRAUN, J. v., BRAUNSDORF, O., AND RÄTH, K., *Ber. deut. chem. Ges.*, **55**, 1666–80 (1922)
21. TODA, K., *Arch. exptl. Path. Pharmakol.*, **146**, 313–26 (1929)
22. KREITMAIR, H., *Klin. Wochschr.*, **15**, 676–78 (1936)
23. KRONER, R., *Klin. Wochschr.*, **15**, 678–81 (1936)
24. HALPERN, B. N., *Compt. rend. soc. biol.*, **126**, 678–82 (1937)
25. MANNICH, C., LESSER, B., AND SILTEN, F., *Ber. deut. chem. Ges.*, **65**, 378–85 (1932)
26. FROMHERZ, K., *Klin. Wochschr.*, **13**, 6–8 (1934)
27. FROMHERZ, K., *J. Pharmacol.*, **60**, 1–13 (1937)
28. CLARK, B. B., SHIRES, E. B. S., JR., CAMPBELL, E. H., AND WELCH, C. S., *J. Pharmacol.*, **66**, 464–78 (1939)
29. CLARK, B. B., AND SHIRES, E. B. S., JR., *J. Pharmacol.*, **70**, 378–87 (1940)
30. U.S. PATENT 1,932,341, *Chem. Abstracts*, **28**, 578 (1934)
31. GERMAN PATENT 586,247, *Chem. Zentr.*, **105**, Part I, 248 (1934)
32. MEIER, R., *Klin. Wochschr.*, **15**, 1403–5 (1936)
33. EINHORN, M., *Am. J. Digestive Diseases*, **5**, 121–25 (1938)
34. MIESCHER, K., AND HOFFMANN, K., *Helv. Chim. Acta*, **24**, 458–65 (1941)
35. U.S. PATENT 2,265,184, *Chem. Abstracts*, **36**, 1737 (1942)
36. U.S. PATENT 2,265,185, *Chem. Abstracts*, **36**, 1737 (1942)
37. HOFFMANN, K., *Helv. Chim. Acta*, **24**, 36E–40E (1941)
38. GILMAN, A., GOODMAN, L., THOMAS, J. M., HAHN, G. A., AND PRUTTING, J. M., *J. Pharmacol.*, **74**, 290–308 (1942)

39. U.S. Patent 2,221,828, *Chem. Abstracts,* **35**, 1935 (1941)
40. Ray, F. E., and Rieveschl, G., Jr., *J. Am. Chem. Soc.,* **65**, 836–39 (1943)
41. Blicke, F. F., and Grier, N., *J. Am. Chem. Soc.,* **65**, 1725–28 (1943)
42. Lewis, J. R., Lands, A. M., and Geiter, C. W., *Federation Proc.,* **2**, 29, 86 (1943)
43. Blicke, F. F., and Feldkamp, R. F., *J. Am. Chem. Soc.* (In press)
44. Yonkman, F. F., Chase, H. F., Lehman, A. J., and Bauer, R., *Federation Proc.,* **2**, 95 (1943)
45. British Patent 396,318, *Chem. Abstracts,* **28**, 577 (1934)
46. Blicke, F. F., and Maxwell, C. E., *J. Am. Chem. Soc.,* **64**, 428–31 (1942)
47. Lindenmeyer, *Berlin. Klin. Wochschr.,* **40**, 1072–73 (1903)
48. Graham, J. D. P., and Lazarus, S., *J. Pharmacol.,* **70**, 165–70 (1940)
49. Quigley, J., *J. Pharmacol.,* **61**, 30–36 (1937)
50. Unna, J., *J. Pharmacol.,* **70**, 179–88 (1940)
51. U.S. Patent 2,230,774, *Chem. Abstracts,* **35**, 3391 (1941)
52. Eisleb, O., *Ber. deut. chem. Ges.,* **74**, 1433–50 (1941)
53. Eisleb, O., *Ber. deut. chem. Ges.,* **74**, 1926 (1941)
54. Eisleb, O., and Schaumann, O., *Deut. med. Wochschr.,* **65**, 967–68 (1939)
55. Climenko, D. R., *Federation Proc.,* **1**, Part II, 15 (1942)
56. Andrews, H. L., *Federation Proc.,* **1**, Part II, 142–43 (1942)
57. Batterman, R. C., *Federation Proc.,* **1**, Part II, 143 (1942)
58. Fellows, E. J., and Cunningham, R. W., *Federation Proc.,* **1**, Part II, 151 (1942)
59. Himmelsbach, C. K., *Federation Proc.,* **1**, Part II, 153 (1942)
60. Clark, B. C., *Federation Proc.,* **2**, 195–201 (1943)
61. Dietrich, H., *Deut. med. Wochschr.,* **65**, 969–70 (1939)
62. Schäfer, F., *Deut. med. Wochschr.,* **65**, 970–72 (1939)
63. Gruber, C. M., Hart, E. R., and Gruber, C. M., Jr., *J. Pharmacol.,* **73**, 319–34 (1941)
64. Pal, J., *Deut. med. Wochschr.,* **39**, 395–98 (1913)
65. Pal, J., *Wien. med. Wochschr.,* **63**, 1050–51 (1913)
66. Pal, J., *Deut. med. Wochschr.,* **40**, 164–68 (1914)
67. Wolfes, O., Dobrowsky, A., and Kreitmair, H., *Merck's Jahresber.,* **44**, 12–25 (1930)
68. Issekutz, B. v., Leinzinger, M., and Dirner, Z., *Arch. exptl. Path. Pharmakol.,* **164**, 158–72 (1932)
69. Issekutz, B. v., Nyary, A., and Botz, B., *Arch. exptl. Path. Pharmakol.,* **164**, 173–78 (1932)
70. Wolfes, O., and Kreitmair, H., *Deut. med. Wochschr.,* **56**, 1702–3 (1930)
71. Cunningham, R. W., and Fellows, E. J., *Federation Proc.,* **1**, Part II, 148 (1942)
72. Macht, D. I., *J. Pharmacol.,* **9**, 287–303 (1917)
73. Macht, D. I., *J. Pharmacol.,* **11**, 389–417, 419–46 (1918)
74. Dunning, B., Jr., Dunning, F., and Reid, E. E., *J. Am. Chem. Soc.,* **58**, 1565–68 (1936)
75. Kreitmair, H., *Arch. exptl. Path. Pharmakol.,* **164**, 509–17 (1932)
76. Slotta, K. H., and Haberland, G., *Z. angew. Chem.,* **46**, 766–71 (1933)
77. Bruckner, V., and Fodor, G. v., *Ber. deut. chem. Ges.,* **71**, 541–49 (1938)

78. Mannich, C., and Walther, O., *Arch. Pharm.*, **265**, 1–11 (1927)
79. Mannich, C., and Falber, M., *Arch. Pharm.*, **267**, 601–9 (1929)
80. Bruckner, V., and Krámli, A., *J. prakt. Chem.*, **145**, 291–300 (1936)
81. Külz, F., and Rosenmund, K. W., with Kayser, E., Schwarzhaupt, O., and Sommer, H., *Ber. deut. chem. Ges.*, **72**, 2161–67 (1939)
82. Külz, F., and Rosenmund, K. W., *Klin. Wochschr.*, **17**, 344–46 (1938)
83. Lux, E., *Klin. Wochschr.*, **17**, 346–47 (1938)
84. Kindler, K., and Peschke, W., *Arch. Pharm.*, **272**, 60–70 (1934)
85. Blicke, F. F., and Monroe, E., *J. Am. Chem. Soc.*, **61**, 91–93 (1939)
86. Blicke, F. F., and Zienty, F. B., *J. Am. Chem. Soc.*, **61**, 93–95, 771–73, 774–76 (1939)
87. Lehman, R. A., *J. Pharmacol.*, **71**, 317–19 (1941)
88. Popkin, R. J., *J. Pharmacol.*, **71**, 320–24 (1941)
89. Mügge, H., *Klin. Wochschr.*, **12**, 381–83 (1933)
90. Gruber, C. M., *J. Pharmacol.*, **56**, 284–89 (1936)
91. Data, J. B., *Dissertation* (University of Michigan, 1941)
92. Lands, A. M., and Geiter, C. W., *Federation Proc.*, **2**, 28 (1943)
93. Geiter, C. W., and Lands, A. M., *Federation Proc.*, **2**, 79–80 (1943)
94. U.S. Patent 2,230,752, *Chem. Abstracts*, **35**, 3390 (1941)
95. U.S. Patent 2,256,434, *Chem. Abstracts*, **36**, 222 (1942)
96. U.S. Patent 2,230,753, *Chem. Abstracts*, **35**, 3391 (1941)
97. U.S. Patent 2,230,774, *Chem. Abstracts*, **35**, 3391 (1941)
98. Issekutz, B. v., Leinzinger, M., and Issekutz, B. v., Jr., *Arch. exptl. Path. Pharmakol.*, **176**, 8–23 (1934)
99. Junkmann, K., *Arch. exptl. Path. Pharmakol.*, **186**, 552–64 (1937)
100. Fromherz, K., *Arch. exptl. Path. Pharmakol.*, **173**, 78–85 (1933)
101. Billman, J. H., and Hidy, P. H., *J. Am. Chem. Soc.*, **65**, 760–61 (1943)
102. Gruber, C. M., Sholten, R., DeNote, A., and Wilson, J. F., *J. Pharmacol.*, **56**, 341–50 (1936)
103. Gruber, C. M., *J. Pharmacol.*, **56**, 432–39 (1936)
104. Reynolds, C., *Proc. Soc. Exptl. Biol. Med.*, **28**, 656–58 (1931)
105. Tatum, A. L., *Physiol Revs.*, **19**, 472–502 (1939)
106. Ehrhart, G., *Z. angew. Chem.*, **43**, 566 (1930)
107. Schultz, F. H., *J. Pharmacol.*, **69**, 300 (1940)
108. U.S. Patent 2,248,018, *Chem. Abstracts*, **35**, 6394 (1941)
109. Winterfeld, K., and Holschneider, K. W., *Arch. Pharm.*, **273**, 315–19 (1935)
110. German Patent 497,907, *Friedlaender*, **16**, 2669 (1931)
111. Kröhnke, F., *Ber. deut. chem. Ges.*, **72**, 2000–9 (1939)
112. British Patent 370,350, *Brit. Chem. Abstracts*, **B**, 786 (1932)
113. Hazard, R., and Moisset de Espanés, E., *Arch. intern. pharmacodynamie*, **59**, 457–60 (1938)
114. Sugihara, H., Nagasawa, S., and Okade, H., *Klin. Wochschr.*, **15**, 751–56 (1936)
115. Raymond, A. L., *J. Am. Pharm. Assoc.*, **32**, 249–55 (1943)

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PHOTOPERIODISM IN PLANTS

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The topic of photoperiodism has received consideration during the last eight years by various reviewers. These have dealt with general reviews of the subject (1 to 5), or with such phases as sex expression (6), nutrition (7, 8), temperature relationships (9), and flowering responses and theories related thereto (10 to 14). These cover the literature up to 1937 rather completely and less completely up to and including 1940.

Photoperiodic responses are varied and involve vegetative extension, tuber formation, resistance to diseases, changes in winter hardiness, initiation of floral primordia and development of flowers, fruits, and seeds, changes in chemical composition and in the activity of enzymes, and many other manifestations. Many of the recent contributions on photoperiodism have dealt with physiological aspects of reproduction in angiosperms, and in the present review the literature will be discussed from that viewpoint. Primarily, papers which have appeared since 1936 have been selected. A fairly complete bibliography of the field may be found in a special section of *Herbage Abstracts*. Since most of the original papers in German and Russian which have appeared since 1940 have not been available to the reviewer, abstracts of them have been used. Wherever possible the original has been consulted.

Photoperiodism in plants may be defined as the responses to changes in the relative length of day and night (1). In this usage, the length of the day is the period from sunrise to sunset. Since the term day has other meanings and since, in experimental work, the period of illumination may not correspond to any of these, the terms photoperiod and dark period have been adopted to designate the length of the periods of exposure of the plants to illumination and to darkness respectively. Photoperiodism is, therefore, the responses to changes in the relative lengths of alternating photoperiods and dark periods, and one alternation (i.e., one photoperiod and the following dark period) is termed a photoperiodic cycle.

Under natural conditions the recurrent alternation of photoperiods and dark periods are in cycles of twenty-four hours, and the length of

the photoperiod is inversely related to the length of the dark period. In the northern latitudes the longest photoperiods of the year (and the shortest dark periods) occur in June, while the shortest photoperiods (and the longest dark periods) are found in December. Some investigators have used the terms "long-day" and "short-day" to describe these respective photoperiodic conditions. Others have preferred the somewhat more specific terms, "long photoperiod" and "short photoperiod." Such terms are relative and have little meaning unless specifically defined. In this discussion the term "long photoperiod" will refer to a twenty-four hour photoperiodic cycle with a photoperiod of sixteen hours or more, accompanied by a dark period of eight hours or less. The term "short photoperiod" will refer to a twenty-four hour cycle with a photoperiod of ten hours or less and a dark period of fourteen hours or more.

Within certain age limits and at certain temperatures, plants which have been growing in photoperiodic conditions which result in vegetative growth may be induced to flower by a short treatment with photoperiodic conditions favorable for flowering even though, after the treatment, the plants are returned to the conditions which originally favored vegetative growth. For example, *Xanthium pennsylvanicum* which has grown vegetatively for months under long photoperiods may be induced to initiate flowers as a result of a treatment with a single short photoperiod even though subsequently grown continuously on long photoperiods. The changes which take place within the plant during this brief photoperiodic treatment and which result in flowering have been called "photoperiodic induction."

Photoperiodic induction may be brought about by photoperiodic cycles other than those twenty-four hours in length. The terms "long day," "short day," "long photoperiod," or "short photoperiod" are often not appropriate to describe these cycles, and the reviewer has followed the practice of using the term "photoinductive cycles" to describe those conditions under which the plants undergo photoperiodic induction and "nonphotoinductive cycles" for those in which the plants tend to remain strictly vegetative.

Classification

Garner & Allard (15), who first advanced the photoperiodic concept, used the flowering response as a basis for the classification of all angiosperms into three groups: long-day plants, short-day plants, and indeterminate plants. The author (16) uses the classification of

Garner & Allard with qualifications. Plants are considered as "long-day" or "short-day" plants if they fit Garner & Allard's classification at some age and temperature, and these particular conditions are assumed to prevail when the responses of such plants are discussed.

Classification of plants into long-day, short-day, and indeterminate groups based upon their photoperiodic responses has been subject to a great deal of criticism. As Thompson has indicated (9) and as has been confirmed by several recent investigations (17 to 20), the temperature prevailing during the investigation may determine whether a plant will or will not be photoperiodically sensitive. In other words, over one temperature range a plant may behave as an indeterminate, and at another temperature it may be either a long-day or a short-day plant. As far as the reviewer is aware, however, there are no undisputed reports of a given plant responding as a typical short-day plant at one temperature and as a typical long-day plant at another temperature.

A great deal of the recent literature has been concerned with the responses of different varieties or strains of a given species. Most of these investigations conclude that as between varieties and strains the responses may vary greatly, and any given species may show a closely graded series of types, ranging from those which are especially sensitive to those relatively insensitive. The response is often correlated with the geographical origin of the strain (21 to 26). In general, however, if a given species contains any strains which are especially sensitive to photoperiod and can be classed without question into either the long-day or short-day group, then all of the other strains of the same species will tend to exhibit responses which would place them in the same class. For example, the varieties or strains of some species may be arranged in a graded series, at one end of which the plants are typical short-day plants and at the other end of which the plants are day-neutral or nearly so (27, 28, 29, *et al.*). Similarly, strains or varieties of other species may be arranged in a graded series at the one end of which are long-day strains and at the other end of which are more or less day-neutral strains (21, 23, 25, 30, *et al.*). The reports that a given species may contain strains, some of which are short-day plants and others of which are long-day plants, are very few (31), and it seems possible that these reports may not represent what has been called "typical" responses. Even a given variety which has been inbred for generations may exhibit heterozygosity in so far as the photoperiodic response is concerned (32, 33). On the other

hand, selection of strains for a particular photoperiodic response is not difficult, and the breeding for a relative degree of sensitivity to photoperiodic changes has been continued successfully (34 to 38).

The paper by Borthwick *et al.* (27) may be used to illustrate the work with different varieties. Of twelve varieties of soybeans examined, seven initiated floral primordia in continuous light within fifty days after planting, and presumably all would have done so had the experiment been continued long enough, since these authors state (39) that the Biloxi variety, which seems to be the most sensitive of all those tested, might nevertheless initiate floral primordia if grown long enough in continuous light. However, the number of nodes with vegetative axillary buds was greater with all varieties in continuous light than in short photoperiod, the number increasing from the least photoperiodically sensitive to the more sensitive strains. Even the Agate variety, which upon casual inspection might be classified as an indeterminate plant since it initiates primordia and develops flowers and fruits over a wide range of day lengths, behaves as a short-day plant during the early stages of its development. In continuous light the first floral primordium is initiated in the axil of the fifth compound leaf, whereas in short photoperiod floral primordia and flower are developed in the axil of the second compound leaf. Thus, with respect to the initiation of floral primordia, the varieties differ primarily in the age which they attain before primordia are initiated on long photoperiods or on continuous light. Similarly, the development of flowers and fruits is affected, and all but one of the varieties behave as short-day plants since they do not produce fruit if the photoperiods are long enough. If the varieties are arranged according to their ability to form fruit in photoperiods of increasing length, almost the same series will be found as was obtained when the plants were arranged according to the age at which floral primordia were initiated in continuous light. Thus, in soybean, short photoperiod stimulates the initiation of flowers as well as the development of flowers and fruits.

It has been indicated by numerous investigators (8, 9, 40, 41, *et al.*) that the process of flowering in plants involves a series of physiological changes. The question naturally arises as to which of the stages are involved in the responses of a particular plant to photoperiodic changes. Eguchi (42) has claimed that plants may fit one classification of Garner & Allard with respect to the early stages of development leading to flowering, and another classification with respect to the subsequent stages. Thus, there are nine possible types of re-

sponses: short day–short day; short day–long day; short day–indeterminate; long day–short day; long day–long day; long day–indeterminate; etc. Loehwing (41) gives a table of these groups with examples of plants belonging to each. Gregory (3) states, "The problem of photoperiodism may, therefore, be considered not as concerning conditions leading to flower formation but as concerning failure to flower." In view of these considerations, it would be helpful in much of the experimental work in this field if the investigators would examine all of their plants by microdissection (43 to 47) to determine what stages of floral development are especially affected by the treatments used. This is especially true when an attempt is made to classify the photoperiodic responses of a given plant. In some cases, plants have been called short-day plants if blossoming occurs at an earlier date when the plants are grown under short photoperiods as compared to long photoperiods even though flowering takes place fairly rapidly under both conditions. Differences of only a few days in the time of flowering have been considered significant. This practice leads to confusion when comparisons are made between such plants and the responses of others which remain strictly vegetative for a long period under one set of conditions and produce flowers and fruits very rapidly when transferred to another.

If we consider the responses of those plants which might be considered "types" representative of the short-day and long-day groups (and these will be the subject of most of the subsequent discussion), the following observations may be made: The age of the plant determines to a large extent its sensitivity to photoperiods (48 to 52). There is a failure to respond by floral initiation to favorable photoperiods until a certain amount of vegetative growth has been obtained; usually one or more foliage leaves are required. In some cases, flowering occurs as the plants grow older in spite of exposure to photoperiodic conditions which at earlier ages favored vegetative growth. The term "photoperiodic induction" implies the stimulation of flowering by exposure to photoinductive cycles and takes place, therefore, only over certain ranges of temperature in plants within certain age limits.

As a result of the interrelationship between photoperiodism and temperature, Whyte has proposed the term thermo-photoperiodic induction (53), and Owen *et al.* (38) have proposed the term photothermal induction. Presumably these terms would include all that is understood by the term photoperiodic induction. Since the latter term

has been so frequently used, it is retained, subject to the implications mentioned above.

Photoperiodic Induction

Whether or not both portions of a photoinductive cycle play determinative roles in photoperiodic induction has interested numerous investigators. Blackman (54) suggested that the length of the dark period was primarily responsible for the photoperiodic response. On the other hand, Lysenko and many other proponents of the theory of "phasic development" (53) proposed that no requirements as to photoperiod are inherent in long-day plants, and that these plants require a continuous day for the completion of one of their developmental phases leading to flowering and can tolerate the daily alternation of light and darkness when the dark period is relatively short. In addition, it was claimed that short-day plants require not a short day but darkness to complete one of the developmental phases leading to flowering and only tolerate daily alternation of darkness and light when the latter is not in excess of a critical duration. Whyte & Oljhovikov (55) claimed that both long-day plants and short-day plants must pass through two phases of development, namely, a dark phase followed by a light phase. The main distinction between the two groups lies in the fact that under natural conditions flowering in short-day plants may be limited by failure in the completion of the dark phase, while in long-day plants it may be limited by failure in the completion of the light phase. The reviewer feels that if the photoperiodic responses are to be made a part of the theory of phasic development, the terms dark phase or light phase are inadequate to cover the changes occurring during photoperiodic induction, and perhaps a term such as "photoperiodic phase" should be substituted. This suggestion is based upon the evidence which indicates clearly that the photoperiodic stimulus to flowering in some and perhaps in all typically short-day plants is brought about during both the light and dark phases of a given cycle or successive cycles of exposure.

The possibility that both phases of a photoinductive cycle are involved was first indicated in the work of Borthwick & Parker with Biloxi soybeans (56). They found that while induction takes place when whole plants are exposed to three short days, it does not take place in comparable plants which are placed in darkness for seventy-two hours. For induction to take place during the three short days the intensity of light during the photoperiod has to be above 100 foot

candles. Moskov (57) with *Perilla ocymoides* found that the minimum daily light period could not fall below three or four hours, and the minimum length of the dark period should not be reduced below eight hours if flowering was to occur. He found (58) with other short-day plants that a cyclic alternation of light and darkness was necessary for induction. The reviewer's own work (59) seems to present clear-cut evidence that both the photoperiod and the dark period of each photoinductive cycle play parts in photoperiodic induction, and that induction may not take place if the dark period of each cycle is not of sufficient duration or if the light period is of insufficient duration or intensity. Since then, Snyder (45), Mann (46), and Cailahjan (60) have reached similar conclusions. A summary of some of this work and the evidence for it has been presented by the reviewer (16). The following is quoted from the latter article with respect to responses of *Xanthium pennsylvanicum* and Biloxi soybean:

> In both plants it would appear that photoperiodic stimulation takes place as a result of exposure to a cyclic alternation of light and darkness in which the light periods are of a certain intensity and duration and the dark periods longer than a definite minimum duration. It appears that determinative reactions take place during both phases of the cycles and also that there is an interaction among them. For convenience, the changes or conditions which arise owing to exposure to light may be designated as A, those owing to darkness as B, and the possible summation or resultant changes related to A and B may be referred to as C. Thus, an interaction between A and B results in C. Such a postulation necessarily implies a carry-over of the effects produced during a photoperiod into a subsequent dark period, or a carry-over of the effects produced during a dark period into the subsequent photoperiod since such must be the case in order for an interaction to take place.
>
> It is assumed that through the medium of C the observable effects such as differentiation of floral primordia, floral development, and the like are manifested. Through the varied expressions of C, therefore, investigations of both A and B would at present be made. Experimental evidence indicates that this is possible. Manipulations of conditions pertaining only to the light period, affecting the expression of C, have been made [examples are given]. Similarly, other expressions of C have been related to conditions during the dark period.
>
> The above use of the symbols A, B, and C has been resorted to as a convenient means of expressing the general photoperiodic responses of both plants. Each may be a whole series of reactions, a complex of substances, or a condition which changes and fluctuates. It is also possible that A, B, and C may represent entirely different things in the two plants.

In the same article, the author pointed out that it is possible that all short-day plants undergo a similar series of reactions during photoperiodic induction, although evidence is not available with respect to

many of them. It seems desirable that a fourth photoperiodic class, "intermediate," proposed by Allard (61), which has been separated as distinct from long-day plants and short-day plants because of the fact that they possess two critical day lengths, should be abandoned and that these plants should be classed with the short-day group. This conclusion is based upon evidence presented above that the short-day plants, *Perilla ocymoides,* Biloxi soybean, and *Xanthium pennsylvanicum,* also possess what might be called two critical day lengths, since they will not flower if the photoperiods are shorter than a certain minimum or if the dark periods are shorter than the critical duration. The major difference between these three plants and the plants described by Allard would seem to lie in the fact that the latter plants require a relatively much longer minimum photoperiod for induction than do the former. Until proof to the contrary is forthcoming, it would seem desirable to describe the photoperiodic conditions under which photoperiodic induction of short-day plants takes place as consisting of photoinductive cycles containing photoperiods of a certain minimum intensity and duration and dark periods of a certain minimum duration. It is recognized, also, that flowering may not take place in certain short-day plants if the photoperiods are longer than a certain maximum (59, 62), while in other short-day plants such is not the case (43, 57).

There seems to be no evidence that long-day plants, in order to be stimulated to flower, require an exposure to darkness. At least, flowering will result when they are exposed throughout their period of growth to continuous illumination (59, 63). Since typical long-day plants do not flower when exposed to short days, the question arises as to whether or not the failure to flower is the result of an exposure to dark periods which are too long or to photoperiods which are too short. All of the evidence seems to point to the fact that failure to flower under natural short days results from the fact that the dark periods are too long, rather than that the photoperiods are too short. For example, flowering will result if short photoperiods are accompanied by short dark periods (1, 62). Exposure to dark periods longer than a certain critical period tends to result in vegetative growth while exposure to dark periods shorter than the critical period results in flowering. However, it also seems that flowering will result if the plants are exposed to photoperiods longer than a certain critical length even though the dark periods are also long. This is illustrated in the work of Allard & Garner (62) with *Rudbeckia bicolor,* a long-day

plant. The plants were grown on cycles varying from ten hours for some to thirty-six for others. Regardless of length, each cycle consisted of a photoperiod of the same duration as the accompanying dark period. The plants tended to remain vegetative on the twenty-four hour cycle, but flowering did take place on both longer and shorter cycles.

Lang & Melchers (64) found that flower formation in the so-called long-day plant *Hyoscyamus niger* could be induced by defoliation whether or not the plants were in long or short day. They postulate that the plants remain vegetative in short day because of inhibitory processes localized in the leaves and taking place or becoming operative only in darkness. This inhibitory action is removed by exposure to low temperatures (65). On the assumption that during long dark periods the decrease in sugars in the leaves might be responsible, they supplied various sugars through infiltration of the leaves to *Hyoscyamus* grown under short-day conditions (66). In nearly every case the infiltration with sugar solutions led to flower formation. Lang (47) found that removal of all of the leaves did not result in flowering if one of the leaves was regrafted to the plant near the growing tip and if this leaf was maintained on short day, thus indicating that injury was not the cause of flowering in defoliated plants. For the plant to remain vegetative under such conditions, however, it was necessary that a close union between the leaf and the plant be established. Lang suggests the possibility that the leaves do not supply to the growing point substances which hinder flower formation, but that from the growing point substances are removed by the leaves which are directly or at preliminary stages, necessary for flower formation.

It is obvious that no general conclusion with respect to the photoperiodic conditions necessary for flowering in long-day plants may be made at this time. It seems possible that other long-day plants might respond in a manner similar to the responses of *Rudbeckia bicolor,* mentioned above, when cycles longer than those of twenty-four hours' duration are used. It would be of particular interest to study the responses of long-day plants on cycles in which length of the dark period remains constant while the length of the photoperiod varies and, vice versa, the responses when the length of the photoperiod remains constant and the length of the dark period varies. It would be of interest to determine whether or not other long-day plants will respond in a manner similar to the responses of *Hyoscyamus niger*.

Comparison of the responses of typical short-day and long-day

plants shows certain similarities and certain contrasts. It seems probable that under natural seasonal conditions flowering in both groups is determined by the length of the "night." In short-day plants flowering seems to be stimulated by long dark period while in long-day plants flowering tends to be inhibited by long dark period. In both groups the effective length of the dark period may be shortened by illumination with light of very low intensity (1, 2, *et al.*). The quality of light effective in shortening the dark period seems to be the same for both groups (67). Intermittent illumination with light of a low intensity will nullify the effect of a long dark period in both groups (59, 68, 69, 70). Whether or not the reactions which occur in short-day plants when they are exposed to darkness and which ultimately lead to stimulation of flowering are the same reactions which occur in long-day plants during exposure to darkness and which inhibit flowering, is not known. Experimentation to elucidate this point would be extremely valuable.

Evidence for a Flowering Hormone

It has been chiefly through the phenomenon of photoperiodism that evidence has been accumulated that at least in certain plants flowering may be stimulated by the action of hormones. This evidence is based upon the fact that the green leaves are the organs which receive the photoperiodic stimulus and that as a result of exposing leaves to photoinductive cycles, a stimulus to flowering is transmitted to the buds. Moskov and Cailahjan have been primarily responsible for the development of this concept, and many other workers have arrived at similar conclusions. The early work in this field has been carefully reviewed by Cholodny (11). Work indicating that the leaves are the locus for the reception of the photoperiodic stimulus has continued.

Evidence (13, 71 to 75) is abundant that the leaves of the plant which are most sensitive to photoperiodic stimulation are neither the very oldest nor the young expanding ones, but are rather those of intermediate age. In short-day plants, exposure of one or more of these leaves to short-day conditions may or may not result in floral initiation depending upon the treatment of the rest of the plant. Hamner & Bonner (43) demonstrated with the short-day plant, *Xanthium*, that fully expanded leaves exposed to long photoperiod exercised an inhibitory effect on the transmission of the stimulus through the stem to which such leaves were attached. They pointed out that this inhibitory effect was localized. Others (39, 76, 77, 78) have also em-

phasized the inhibitory nature in short-day plants of old leaves exposed to long photoperiods. If the old leaves are placed in complete darkness instead of long photoperiods much of this inhibition disappears (79). Similarly, if the buds in the vicinity of treated leaves are removed the stimulus is received by more distant buds with greater force (43).

The rate of transfer of the stimulus from the leaves to the terminal growing point seems to be relatively slow (43, 80). Evidence is accumulating that living cells are involved in its transmission (80 to 83). Local applications of low temperature to the petioles of leaves or to the stem axes between the leaves which serve as a source of the stimulus and the meristem which is expected to respond greatly delays the transmission of the stimulus (80, 84). Applications of narcotics have similar effects (80). There seems little question but that the stimulus may be transferred from one plant to another through a graft union, and the graft partners need not necessarily be of the same species or photoperiodic classification (11, 16). Hamner & Bonner (43) reported that the stimulus may be transferred from one plant to another across a "diffusion contact" in which the injured surfaces of the two plants were separated by lens paper, and in which presumably there was no tissue contact between the two plants. This possibility has been questioned by Withrow (83) who claims that in such cases transmission of the stimulus occurs only when growth of cells through the lens paper from one plant to another occurs, although admittedly the union between the two plants is very slight.

Proof of the existence of a hormone has not yet been forthcoming, since no one has successfully extracted a substance from photoperiodically-induced leaves, identified and synthesized it, and reapplied it to vegetative plants, thereby inducing floral formation. In fact, so far as is known no one has obtained an active extract from flowering plants which will result in floral initiation in vegetative plants.

Miscellaneous Topics and Theories

Cholodny (11) has advanced the theory that a separate hormone for flowering may not exist but rather that the plant growth hormones, the auxins, may be involved in the transfer of the flowering stimulus from one part of the plant to another. Some investigators seem inclined to agree (85), and others have disagreed (7, 86). Loehwing (87) emphasizes the increased transpiration and marked changes in water relations which occur during photoperiodic induction. He pos-

tulates that, as a result of these changes in the internal water balance, changes occur in solute constituents of the sap, particularly in mineral elements, and these result in redistribution of nutrient elements throughout the plant. He feels such changes are related to the initiation of flowering. The fact that photoperiodic induction of leaves is dependent upon photosynthesis is indicated by the work of Parker & Borthwick (88), who found that flowering did not occur unless the plants were supplied with carbon dioxide during exposure to photoinductive cycles. Harder & Witsch (89) found that induction of an individual leaf was dependent upon carbon dioxide supply even though favorable conditions for photosynthesis prevailed for the rest of the plant. Changes in the photosynthetic activity (90, 91) and in glutathione content (92) of leaves during induction have been reported. Variations in chlorophyll content during photoperiodic responses have been followed (93), but plants with very low chlorophyll content as a result of reduced iron supply nevertheless give a characteristic photoperiodic response (60). Struckmeyer (94) found that during photoperiodic induction the cambial activity of the stems decreased greatly, but Pasrev *et al.* (95, 96, 97) found an increased cambial activity during induction. Little correlation between enzyme activity and photoperiodic induction has been found (98, 99, 100). Studies of vitamin content in relation to photoperiodism have been few (101, 102, 103).

Whyte & Oljhovikov (55) postulate that both long- and short-day plants undergo a succession of ecologico-physiological changes and that irrespective of the photoperiodic group, flower-inducing substances (florigen) are manufactured under the influence of light in the leaves of all plants during the so-called photophase. This interpretation is difficult to reconcile with the results obtained with *Xanthium* (40) in which flowering was induced by placing the plant in continuous darkness. Apparently, in this latter case, exposure to light after a long dark period was not essential for floral initiation.

Grainger (104) proposed the hypothesis that the initiation of flowering was influenced by the rate of translocation of food materials from the leaves to the rest of the plant. In the long-day plants examined, translocation of food materials began soon after the plants were placed in darkness, whereas in short-day plants translocation of carbohydrates did not begin until after the plants had been exposed to a relatively long period of darkness. He postulates that short-day plants in order to flower need a relatively long dark period to afford oppor-

tunity for the translocation of food materials from the leaves. There seems little supporting evidence for this theory.

Several investigators (105 to 112) have found that a number of plants which respond to vernalization also respond as long-day plants after the vernalization period, although photoperiodic treatment before vernalization is noneffective. In all these cases, the vernalization consisted of treatment at low temperatures for appreciable periods of time. As far as the reviewer is aware, there are no cases on record where plants which require a treatment with low temperature behave as typical short-day plants subsequent to the low-temperature treatment.

LITERATURE CITED

1. Garner, W. W., Chap. XIX, *Biological Effects of Radiation* (McGraw-Hill Book Co., New York, 1936)
2. Burkholder, P. R., *Botan. Rev.*, **2**, 1–52, 97–168 (1936)
3. Gregory, F. G., *Sci. Hort.*, **4**, 143–54 (1936)
4. Bowman, F. T., *J. Australian Inst. Agr. Sci.*, **4**, 25–32 (1938)
5. Tincker, M. A. H., *Sci. Hort.*, **6**, 133–49 (1938)
6. Loehwing, W. F., *Botan. Rev.*, **4**, 581–625 (1938)
7. Murneek, A. E., *Missouri Agr. Expt. Sta. Research Bull.*, **268**, 84 pp. (1937)
8. Murneek, A. E., *Growth*, **3**, 295–315 (1939)
9. Thompson, H. C., *Proc. Am. Soc. Hort. Sci.*, **37**, 672–79 (1939)
10. Cailahjan, M. H., *Compt. rend. acad. sci. U.R.S.S.*, **16**, 227–30 (1937)
11. Cholodny, N. G., *Herbage Revs.*, **7**, 223–47 (1939)
12. Anonymous, *Herbage Revs.*, **7**, 27–32, 94–99, 181–89, 265–74 (1939); **8**, 83–93 (1940)
13. Ullrich, H., *Ber. deut. botan. Ges.*, **57**, 40–52 (1939)
14. Adler, F., *Forschungsdienst*, **9**, 332–67 (1940)
15. Garner, W. W., and Allard, H. A., *J. Agr. Research*, **18**, 553–606 (1920)
16. Hamner, K. C., *Cold Spring Harbor Symposia Quant. Biol.*, **10**, 49–59 (1942)
17. Knott, J. E., *Cornell Univ. Agr. Expt. Sta. Mem.*, **218** (1939)
18. Roberts, R. H., and Struckmeyer, B. E., *J. Agr. Research*, **59**, 699–709 (1939)
19. Murneek, A. E., *Botan. Gaz.*, **102**, 269–79 (1940)
20. Celjadinova, A. I., *Compt. rend. acad. sci. U.R.S.S.*, **31**, 55–57 (1941)
21. Beljdenkova, A. F., *Sovet. Botan.*, **4**, 47–67 (1940)
22. Elladi, E. V., *Izvest. Akad. Nauk S.S.S.R.*, Ser. Biol., No. **3**, 371–88 (1939)
23. Laibach, F., *Wien. Landwirth. Zeit.*, **90**, 49–50 (1940)
24. Sivori, E. M., *Rev. argentin. Agron.*, **7**, 185–90 (1940)
25. Goodwin, R. H., *Proc. Rochester Acad. Sci.*, **8**, 22–27 (1941)
26. Olmsted, C. E., *Bull. Ecol. Soc. Am.*, **23**, 72–73 (1942)
27. Borthwick, H. A., and Parker, M. W., *Botan. Gaz.*, **101**, 341–65 (1939)
28. Rjabov, I. E., *Herbage Abstracts*, **11**, 442 (1941)
29. Stoletova, E. A., *Herbage Abstracts*, **11**, 477 (1941)
30. Allard, H. A., *J. Agr. Research*, **63**, 55–64 (1941)
31. Nikolaenko, E. I., *Compt. rend. acad. sci. U.R.S.S.*, **30**, 353–55 (1941)
32. Razumov, V. I., *Jarovizacija*, **11**, 74–82 (1937)
33. Kiricenko, F. G., and Bassarskaja, M. A., *Jarovizacija*, **11**, 83–88 (1937)
34. Razumov, V. I., *Jarovizacija*, **25**, 107–14 (1939)
35. Vasiljev, L. V., *Herbage Abstracts*, **11**, 112 (1941)
36. Almeida, J. M. d., *Herbage Abstracts*, **13** (1943)
37. Demicinskaja, E. N., *Herbage Abstracts*, **11**, 118 (1941)

38. Owen, F. V., Carsner, E., and Stout, M., *J. Agr. Research,* **61**, 101–24 (1940)
39. Heinze, P. H., Parker, M. W., and Borthwick, H. A., *Botan. Gaz.,* **103**, 518–30 (1942)
40. Hamner, K. C., *Botan. Gaz.,* **99**, 615–29 (1938)
41. Loehwing, W. F., *Science,* **90**, 552–55 (1939)
42. Eguchi, T., *Proc. Imp. Acad. (Tokyo),* **13**, 332–33 (1937)
43. Hamner, K. C., and Bonner, J., *Botan. Gaz.,* **100**, 388–431 (1938)
44. Borthwick, H. A., and Parker, M. W., *Botan. Gaz.,* **99**, 825–39 (1938)
45. Snyder, W. E., *Botan. Gaz.,* **102**, 302–22 (1940)
46. Mann, L. K., *Botan. Gaz.,* **102**, 339–56 (1940)
47. Lang, A., *Naturwissenschaften,* **30**, 590–91 (1942)
48. Purvis, O. N., and Gregory, F. G., *Ann. Botany,* **1**, 569–91 (1937)
49. Botvinovskii, V. V., *Herbage Abstracts,* **9**, 682 (1939)
50. Moskov, B. S., *Compt. rend. acad. sci. U.R.S.S.,* **22**, 460–63 (1939)
51. Borthwick, H. A., and Parker, M. W., *Botan. Gaz.,* **100**, 245–49 (1938)
52. Miroljubov, K. S., *Izvest. Akad. Nauk S.S.S.R.,* **4**, 463–73 (1940)
53. Whyte, R. O., *Biol. Revs. Cambridge Phil. Soc.,* **14**, 51–87 (1939)
54. Blackman, V. H., *Nature,* **137**, 931–34 (1936)
55. Whyte, R. O., and Oljhovikov, M. A., *Chronica Botanica,* **5**, 327–31 (1939)
56. Borthwick, H. A., and Parker, M. W., *Botan. Gaz.,* **100**, 374–87 (1938)
57. Moskov, B. S., *Compt. rend. acad. sci. U.R.S.S.,* **22**, 456–59 (1939)
58. Moskov, B. S., *Sovet. Botan.,* **4**, 32–45 (1940)
59. Hamner, K. C., *Botan. Gaz.,* **101**, 658–87 (1940)
60. Cailahjan, M. H., *Compt. rend. acad. sci. U.R.S.S.,* **31**, 945–48 (1941)
61. Allard, H. A., *J. Agr. Research,* **57**, 775–89 (1938)
62. Allard, H. A., and Garner, W. W., *J. Agr. Research,* **63**, 305–30 (1941)
63. Naylor, A. W., *Botan. Gaz.,* **102**, 557–75 (1941)
64. Lang, A., and Melchers, G., *Naturwissenschaften,* **29**, 82–83 (1941)
65. Lang, A., *Biol. Zentr.,* **61**, 427 (1941)
66. Melchers, G., and Lang, A., *Naturwissenschaften,* **30**, 589–90 (1942)
67. Withrow, R. W., and Withrow, A. P., *Plant Physiol.,* **15**, 609–24 (1940)
68. Katunskij, V. M., *Compt. rend. acad. sci. U.R.S.S.,* **3**, 303–4 (1936)
69. Hume, E. P., *Proc. Am. Soc. Hort. Sci.,* **37**, 1059–65 (1940)
70. Arthur, J. M., and Harvill, E. K., *Contrib. Boyce Thompson Inst.,* **11**, 93–103 (1940)
71. Moskov, B. S., *Trudy Priklad. Botan., Genetike i Selektsii, A,* **21**, 145–56 (1937)
72. Borthwick, H. A., and Parker, M. W., *Botan. Gaz.,* **101**, 806–17 (1940)
73. Psarev, G. M., *Compt. rend. acad. sci. U.R.S.S.,* **17**, 435–37 (1937)
74. Naylor, A. W., *Botan. Gaz.,* **103**, 342–53 (1941)
75. Gerhard, E. *J. Landw.,* **87**, 161 et seq. (1940)
76. Moskov, B. S., *Compt. rend. acad. sci. U.R.S.S.,* **31**, 699–701 (1941)
77. Cailahjan, M. H., and Jarkova, L. M., *Trudy Timirjazev Inst. Physiol.,* **2**, 133–43 (1938)
78. Loehwing, W. F., *Chronica Botanica,* **4**, 497–98 (1938)
79. Moskov, B. S., *Compt. rend. acad. sci. U.R.S.S.,* **31**, 161–62 (1941)

80. Cailahjan, M. H., *Compt. rend. acad. sci. U.R.S.S.*, **31**, 949–52 (1941)
81. Moskov, B. S., *Compt. rend. acad. sci. U.R.S.S.*, **15**, 211–14 (1937)
82. Cailahjan, M. H., *Compt. rend. acad. sci. U.R.S.S.*, **18**, 607–14 (1938)
83. Withrow, A. P., and Withrow, R. B., *Botan. Gaz.*, **104**, 409–16 (1943)
84. Borthwick, H. A., Parker, M. W., and Heinze, P. H., *Botan. Gaz.*, **102**, 792–800 (1941)
85. Psarev, G. M., and Veselovskaja, H. A., *Compt. rend. acad. sci. U.R.S.S.*, **30**, 844–47 (1941)
86. Cailahjan, M., and Zdanova, L. P., *Compt. rend. acad. sci. U.R.S.S.*, **19**, 107–11 (1938)
87. Loehwing, W. R., *Science*, **92**, 517–20 (1940)
88. Parker, M. W., and Borthwick, H. A., *Botan. Gaz.*, **102**, 256–68 (1940)
89. Harder, R., and Witsch, H. v., *Naturwissenschaften*, **29**, 770–71 (1941)
90. Katunskii, V. M., *Izvest. Akad. Nauk S.S.S.R.*, Ser. Biol., No. **1**, 85–102 (1939)
91. Bode, O., *Planta*, **33**, 278–89 (1942)
92. Ivanov, S. M., *Compt. rend. acad. sci. U.R.S.S.*, **28**, 736–38 (1940)
93. Celjadinova, A. I., *Izvest. Inst. Lesgafta*, **20**, 127–34 (1938)
94. Struckmeyer, B. E., *Botan. Gaz.*, **103**, 182–91 (1941)
95. Psarev, G. M., *Compt. rend. acad. sci. U.R.S.S.*, **20**, 731–34 (1938)
96. Psarev, G. M., and Neuman, N. F., *Compt. rend. acad. sci. U.R.S.S.*, **29**, 497–99 (1940)
97. Psarev, G. M., *Compt. rend. acad. sci. U.R.S.S.*, **28**, 537–39 (1940)
98. Hibbard, A., *Missouri Agr. Expt. Sta. Research Bull.*, **271**, 48 pp. (1937)
99. Cailahjan, M. H., *Trudy Timirjazev Inst. Physiol.*, **2**, 95–106 (1938)
100. Kovalenko, V. G., *Herbage Abstracts*, **9**, 185 (1939)
101. Bonner, J., *Plant Physiol.*, **15**, 319–25 (1940)
102. Zhdanova, L. P., *Compt. rend. acad. sci. U.R.S.S.*, **32**, 584–87 (1941)
103. Reid, M. E., *Bull. Torrey Botan. Club*, **69**, 204–20 (1942)
104. Grainger, J., *Ann. Applied Biol.*, **25**, 1–19 (1938)
105. Eremenko, V. T., *Herbage Abstracts*, **8**, 1856 (1938)
106. Mackov, F., Simanskii, N., and Trigubenko, M., *Herbage Abstracts*, **8**, 1848 (1938)
107. Larose, E., and Vanderwalle, R., *Gemblaux, Inst. Agron. l'état*, Bull. **7**, 149–62 (1938)
108. Reimers, F. E., *Compt. rend. acad. sci. U.R.S.S.*, **20**, 603–8 (1938)
109. Hartisch, J., *Pflanzenbau*, **15**, 265–88 (1939)
110. Kruzilin, A. S., *Herbage Abstracts*, **11**, 111 (1941)
111. Sorokin, K. A., *Compt. rend. acad. sci., U.R.S.S.*, **35**, 51–54 (1941)
112. Arthur, J. M., and Harville, E. K., *Contrib. Boyce Thompson Inst.*, **12**, 111–17 (1941)

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CHLOROPLAST PIGMENTS

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With the rise and expansion of organic chemistry in the past century, man has produced, from coal and petroleum, many of the carbonaceous materials demanded by industry (1). He has been attracted from agricultural to industrial pursuits, from a rural to an urban mode of living, but, though it is often forgotten, he has not yet divorced himself from dependence upon the living plant for myriad carbon compounds.

From the simplest substances, carbon dioxide, water, and sunlight, autotrophic plants produce enormous quantities of organic matter. For equal areas on the earth's surface, the productivity of marine and of lacustrine plants often compares favorably with that of land plants (2 to 11). Synthesis of all this diverse vegetable material hinges upon photochemical reactions that take place within the green parts of plants.

Of the thousands of scientific workers throughout the world, not more than a few score are engaged in studies of the special reactions by which plants capture sunlight and synthesize the complex carbon compounds required as foods by all other organisms. It is not surprising that, today, so little is known regarding the specific steps involved in this complex, vital process. "Freedom from want," as measured by an adequate food supply (12), is an ideal (13) based upon syntheses that remain unknown to scientists and politicians alike. In the future, advantages will accrue to the peoples most adept at the production, modification, and utilization of carbonaceous vegetable matter.

Function of Chloroplast Pigments

Pigments absorb the light utilized by plants for the synthesis of organic matter. In the spectral regions where the pigments do not absorb light, there is no utilization of the incident radiation. When thin layers of green plant tissue are exposed to bright monochromatic light, the amount of energy fixed at a given wave length is proportional to the absorption capacity of the pigments (14, 15).

Plants that lack their normal complement of pigments can not utilize radiant energy. Yet these same pigment-deficient organisms grow and bear fruit when fed organic material (16, 17). Apparently the pigments serve primarily for the absorption and transfer of the

radiant energy utilized by the plant. They are not essential for the multifarious transformations of the organic compounds produced by the photochemical syntheses.

Each investigator, following his own skills and often ignoring those of others, has sought to answer numerous questions regarding the nature and mode of action of the various pigments contained within the photosynthetic apparatus. Plants have been dissected and examined microscopically. Environmental conditions affecting the synthesis and degradation of the pigments have been studied in detail. The pigments have been extracted from various species of plants and separated from one another by ingenious physical and chemical procedures. Physical and chemical properties of the purified pigments have been determined with great care and have then been compared with the properties of the pigments as they exist in the living plant. Special attention has been given to the spectral properties of the purified pigments in relation to their photosynthetic activity in monochromatic light. These new results have aided precise description of the photosynthetic apparatus. They have indicated the roles played by specific pigments. They are forming a foundation for interpretation of the mechanism of the photochemical process, and, in conjunction with genetic, ecologic, and edaphic studies, they are aiding improvement of the yield and the quality of numerous agricultural products (18).

Properties of Chloroplasts

Photosynthetically active pigments occur within the cells in special bodies, the chloroplasts, in which the products of photosynthesis first accumulate (19). In one class of plants, the blue-green algae (Cyanophyta), distinct pigmented structures are not readily visible, the pigments appearing to be rather uniformly distributed about the periphery of the protoplast (14, 20). Under the electron microscope, chloroplast material itself appears to be heterogeneous (21). Chloroplasts removed from the plant cells are able to carry on photosynthesis, but this capacity is rapidly lost (22, 23). Addition of ferric salts to the medium containing the isolated chloroplasts accelerates liberation of oxygen without simultaneous assimilation of carbon dioxide (24, 25, 26). The presence of weakly reducing substances such as fructose also facilitates photosynthesis in isolated chloroplasts (27).

In chloroplasts separated from the cells of higher plants, the pigments are intimately associated with fats and proteins, but the compo-

sition of the chloroplasts is by no means constant (28 to 40). For whole, deep green *Chlorella* cells, the pigment content and the ratio of pigment to nitrogen may exceed values for the isolated chloroplasts of higher plants.

In many hybrids, mutants, bud sports, etc., the plastids may be colorless or yellow. In seedlings germinated in the dark, the plastids are usually yellow, becoming green only upon exposure to light. The repeated claim that leaves of yellow etiolated bean seedlings do not contain carotenoid pigments (41) may be attributed to the fact that the small quantity of these pigments undergoes rapid oxidation to colorless products when the plant cells are killed under conditions undestructive to enzymes (42, 43).

As many fruits and flowers mature, the green pigments present in the plastids during the early stages of development gradually disappear, being superseded by yellow, carotenoid pigments, some of which may not have been present in the green tissue. Formation of these yellow pigments with the simultaneous loss of chlorophyll is commonly an irreversible process (44). Xanthophylls in these yellow tissues frequently occur in the form of esters, whereas the xanthophylls of the green tissues are unesterified (42). So far as is known, plant material containing only yellow plastids lacks photosynthetic activity, but does show phototropic responses (16), an indication that some pigments may cause secondary photochemical effects (45, 46).

Yellow plastid pigments are of considerable technological importance. Some of them are valuable sources of vitamin A (47). Many of them impart the characteristic red and yellow colors to numerous fruits and vegetables, and to some animal products; hence, they affect the appearance and salability of all these materials. The quantity of yellow pigment is also used as a measure of the quality of many fresh and preserved foods (48). A few of these yellow pigments serve as activators (hormones) for sexual responses of certain unicellular algae (49). Others play a role in vision (50).

Pigments of the Chloroplasts

Analytical investigations, based primarily upon Tswett's chromatographic or columnar adsorption technique (28, 51, 52, 53) and upon spectral absorption methods (54, 55), have revealed numerous chlorophylls, carotenes, and xanthophylls in various plant species. Some of these pigments with information concerning their occurrence in plants are recorded in Table I (56 to 59). These results, which are based

upon examination of relatively few species, may be subject to minor revisions and additions when the investigations are extended.

TABLE I

THE OCCURRENCE OF CHLOROPLAST PIGMENTS IN VARIOUS GROUPS OF PLANTS*

Pigment	Higher Plants	Green Algae	Euglenophyceae	Brown Algae	Diatoms	Dinoflagellates	Yellow-Green Algae	Red Algae	Blue-Green Algae
Chlorophylls									
Chlorophyll *a*	++	++	++	++	++	++	++	++	++
Chlorophyll *b*	++	++	++	−	−	−	−	−	?
Chlorophyll *c*	−	−	−	++	++	++	−	−	−
Chlorophyll *d*	−	−	−	−	−	−	−	++	−
Xanthophylls									
Cryptoxanthin	++	++							
Lutein	++	++	−	−	−	−	−	+	
Zeaxanthin	++	++	−	−	−	−	−		
Violaxanthin	++	++		++	−	−			
Flavoxanthin	++	++		+					
Neoxanthin	++	++			−	−			
Fucoxanthin	−	−	−	++	++	−	−	?	−
Neofucoxanthin A	−	−	−	++	++	−	−	?	−
Neofucoxanthin B	−	−	−	++	++	−	−	?	−
Diatoxanthin	−	−	−	?	++	−	−		
Diadinoxanthin	−	−	−	?	++	++	−		
Dinoxanthin	−	−	−	−	−	++	−		
Neodinoxanthin	−	−	−	−	−	++	−		
Peridinin (sulcatoxanthin)	−	−	−	−	−	++	−		
Myxoxanthophyll	−	−	−	−	−	−	−	−	+
Unnamed xanthophylls			+	+			+		
Carotenes									
β-Carotene	++	++	++	++	++	++	++	++	++
α-Carotene	+	+	−	−	−	−	−		
ε-Carotene	−	−	−	−	+	−		−	
Flavacin									+
Proteinaceous pigments									
Phycoerythrin	−	−	−	−	−	−	−	+	+
Phycocyanin	−	−	−	−	−	−	−	+	+

* ++ Indicates the presence of the pigment in most or in all of the plants examined.
\+ Indicates its presence in some of the plants examined.
− Indicates its absence.
? Indicates small quantities that may have come from contamination of the source by other organisms.
A blank space indicates that a thorough search was not made for the pigment.

With the exception of the highly specialized green and purple sulfur bacteria which contain bacteriochlorophyll and unique carotenoid pigments (60), autotrophic plants, ranging from the algae to the compositae, contain chlorophyll *a,* β-carotene, and several xanthophylls. In some plants these pigments are often accompanied by smaller quantities of other chlorophylls and occasionally by other carotenes. In a few plants, they are associated with water-soluble, proteinaceous pigments.

Of the several groups of chloroplast pigments, the xanthophylls are subject to the greatest variation. Different xanthophylls preponderate in plants belonging to different classes. Many plants do not contain a single xanthophyll in common.

Chlorophyll *b* occurs in green algae and in higher plants. Its reported occurrence in the marine diatom, *Nitzschia closterium* (61), has not been confirmed (57, 59). This chlorophyll is found in largest amounts in plants that form starch as a product of photosynthesis. This fact has led to the presumption that chlorophyll *b* plays a specific role in the formation of the polysaccharide (45, 46). It is of interest in connection with fluorescence phenomena that the wave length of the fluorescent light from chlorophyll *b* is less than that from chlorophyll *a* whereas the wave length of the fluorescent light from chlorophyll *d,* the additional chlorophyll of red algae, is greater than that from chlorophyll *a.*

Chlorophyll *c,* also called chlorofucine and chlorophyll γ, and frequently reported as a post-mortem product, is now found to be a normal constituent of algae belonging to three important groups, diatoms, dinoflagellates, and brown algae (57, 59). This chlorophyll *c,* first reported in brown algae in 1874, bears no relation to the reputed chlorophyll *c* of higher plants, claims to the discovery of which have been retracted (62). Owing to the abundance and wide distribution of diatoms, dinoflagellates, and brown algae in the sea, and because of the restricted occurrence of chlorophyll *b* in algae, chlorophyll *c* must rank with chlorophyll *b* in respect to abundance, to geographical distribution, and to possible importance in the photosynthetic apparatus of plants.

Reports that lutein, the principal xanthophyll of higher plants and green algae, and zeaxanthin occur in diatoms and in brown algae (7, 61, 63 to 68) were not confirmed by the results summarized in Table I. Diatoxanthin of diatoms, which is adsorbed next above the chlorophyll *a* on columns of sugar (28), is chromatographically very similar

to zeaxanthin. With absorption maxima at 453 and 481 mμ in ethanol and with a characteristic spectral absorption curve almost identical with that of zeaxanthin, diatoxanthin might readily have been mistaken for zeaxanthin. Diadinoxanthin, adsorbed as a yellow band between diatoxanthin and the striking orange band of fucoxanthin (28), is considerably more adsorbed than the spectroscopically similar lutein with which it could have been confused.

The common, stable fucoxanthin or fucoxanthin *a* of diatoms and brown algae occurs in two labile, interconvertible forms (67, 69). Instead of calling these labile isomers fucoxanthins *b* and *c* (28), it is now proposed to call them neofucoxanthins A and B in accordance with the nomenclature employed with other interconvertible carotenoids (70). Because diatoms and brown algae are the principal photosynthetic plants over a large fraction of the earth's surface, fucoxanthin must comprise a considerable proportion of the xanthophylls in the world's vegetation.

Peridinin, a xanthophyll found in quantity long ago in the abundant and widely distributed marine dinoflagellates (71, 72), has also been observed in fresh water dinoflagellates (7, 56) and in an alga that grows symbiotically in a sea anemone (56). It appears to be the same pigment subsequently named sulcatoxanthin (73). If the symbiotic algae are cryptomonads, as has been suggested, then the free-living cryptomonads might be expected to contain this xanthophyll.

Dinoxanthin, which is adsorbed below the red-orange peridinin and just above the diadinoxanthin in extracts of dinoflagellates, is spectroscopically similar to violaxanthin and taraxanthin. Like taraxanthin and unlike violaxanthin, it is not colored blue by concentrated hydrochloric acid in ether. It is chromatographically distinct from each of these pigments.

The phylloxanthin of leaves and of brown algae (67), the violaxanthin *b* of leaves (42) and the violaxanthin of pansies are chromatographically and spectroscopically identical pigments. In spite of the fact that phylloxanthin was the name first used for this pigment, the wide acceptance of the name violaxanthin, particularly in the extensive chemical literature, makes its continued use desirable.

In addition to the pigments reported in Table I, a number of other xanthophylls have been observed in the chloroplasts of various plants (63 to 67). Taraxanthin has been reported in green algae (74) and both taraxanthin (67) and fucoxanthin in a few species of red algae (75). A xanthophyll variously known as myxorhodin, phyco-

xanthin, myxoxanthophyll, and aphanizophyll, has been found in the blue-green algae along with calorhodin-α or aphanin and with calorhodin-β or aphanicin. Lutein and the carotene-like pigment flavacin were also observed in these algae (67). Eloxanthin occurs in the pond-weed *Elodea canadensis* (76). The yellow-green alga *Tribonema bombycinum* contains a number of xanthophylls most of which appear to be different from those observed in other plants (56). With respect to its chloroplast pigments, *Tribonema* is not closely related to diatoms, although it has been classed with them (77). Some of the carotenoids isolated from heated plant material may be alteration products of the native pigments (77a).

A red, proteinaceous, water-soluble pigment, phycoerythrin, occurs with the green and yellow pigments in the chloroplasts of most red algae. A similar blue pigment, phycocyanin, has been found in some red algae and in most blue-green algae. Some blue-green algae also contain phycoerythrin along with the phycocyanin; some contain neither of these proteinaceous pigments. There are indications that several similar phycocyanins and phycoerythrins may occur in the various red and blue-green algae (78).

Chloroplasts of all types of photosynthetic organisms do not contain a single pigment in common. Because plants belonging to the same class usually contain the same chloroplast pigments, taxonomic and phylogenetic relationships may be predicted from the nature of these pigments (56, 63, 64, 65).

Pigment Formation in the Chloroplasts

Many conditions influence the development of green and yellow pigments in the chloroplasts (79 to 90). In seedlings of most plants light, oxygen, and moderate temperature are necessary for the formation of the normal amount of green and yellow pigments. As a rule, other conditions being comparable, more pigments are formed in light of low intensity than in light of high intensity (56, 83, 86).

Relative proportions between the various yellow pigments of algae vary with changes in the conditions of culture. Grown in light from neon fluorescent lamps, the diatom *Nitzschia closterium* contained more diadinoxanthin relative to fucoxanthin than the same organism grown in light from "snow white" fluorescent lamps. The green alga *Chlorella pyrenoidosa* grown in continuous light of low intensity contained more α-carotene than β-carotene, whereas similar material

grown in continuous light of high intensity yielded more β-carotene than α-carotene (56).

Under natural conditions, there is little indication of diurnal variation in the chlorophyll content of higher plants (84, 85). Plants growing at different altitudes also show little variation in pigment content. With deficiencies in mineral nutrients, the green parts of many plants become pale yellow (91, 92). Pyrrole compounds do not alleviate chlorosis due to deficiency of iron salts (93).

Temperature during the period of illumination relative to that during the period of darkness has a pronounced effect upon the development of pigments in land plants. Species native to temperate climates develop little green pigment if the temperature of the light period is low, 13°, and that of the dark period is high, 24°. Plants native to subtropical regions show less response to variations in the temperature of the light and dark periods (94). All these facts show how delicately plant species and their complex photosynthetic apparatus are adjusted to various environmental conditions (6, 95).

Genetic changes in plants may retard or prevent development of the chloroplast pigments, many yellow, chlorophyll-deficient mutants being found under natural conditions. This capacity for variation of the chloroplast pigments when considered with the fact that no viable mutant nor any species has yet been found in which chlorophyll is not accompanied by yellow pigments suggests that the combination of green and yellow pigments is in some way essential to the development of the plant. It may be that both the green and yellow pigments play separate vital roles, that they play joint vital roles, or that their formation is coupled with the formation of other materials, such as enzymes (96), that play vital roles.

Properties of Chloroplast Pigments

Although belonging to dissimilar chemical types, chloroplast pigments possess many properties in common. All are fluorescent. All owe their color to systems of conjugated double bonds. Most are readily oxidizable, especially upon irradiation. Both chlorophylls and carotenoids yield interconvertible isomers, but usually only the most stable of these are found in the green parts of plants.

Molecular structure and isomerization.—Extensive chemical investigations of the chlorophylls that led to establishment of their molecular structure have been carefully reviewed by the workers most active in this field (97 to 101). Thus far these molecular structures have

prompted numerous divergent speculations regarding the possible role of the green pigments in the photosynthetic process (97 to 104).

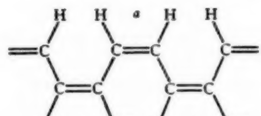
When solutions of chlorophylls *a* and *b* are heated, about 20 per cent of each pigment is converted reversibly into a spectrally similar, less adsorbed isomer called chlorophyll *a′* and chlorophyll *b′*, respectively. Pheophytins derived from the labile isomers differ but slightly from pheophytins prepared from the more stable, common chlorophylls. The structural difference between each of the common chlorophylls and its less stable isomer probably does not, therefore, involve the magnesium atom (105). The slowness of the interchange of radioactive hydrogen and magnesium ions with magnesium in the chlorophyll molecule (106, 107, 108) indicates that isomerization of the chlorophylls does not involve liberation and recombination of the magnesium.

Chlorophyll *d,* also a magnesic pigment, yields one spectrally similar isomer, chlorophyll *d′*, and two other isomers, isochlorophyll *d* and isochlorophyll *d′*, that resemble chlorophyll *a* and chlorophyll *a′*. Each of these four isomers in the chlorophyll *d* series yields a distinct pheophytin but only the isopheophytins *d* and *d′* can be reconverted to the corresponding chlorophylls, isochlorophylls *d* and *d′*, by the action of methyl magnesium iodide. Remarkably close resemblance between the spectral absorption curves of chlorophyll *a* and of isochlorophyll *d* and between the curves of pheophytin *a* and isopheophytin *d* suggests that all these pigments contain similar chromophoric structures. When treated with alkali and acid, the four pigments of the chlorophyll *d* series yield a single product that is spectrally quite different from the analogous product yielded by the chlorophyll *a* compounds (109).

Rapid isomerization of the chlorophylls in solution suggests that the allomerization process may involve isomerization as well as oxidation reactions. If pure preparations of chlorophylls are desired, great care must be exercised in the extraction and purification of these pigments in order to prevent alteration by enzymatic hydrolysis (106) and by isomerization.

In the past five years considerable progress has been made in the elucidation of the spatial configurations about the double bonds in the carotenoid pigments. When solutions of the common stable or *trans* pigments are heated or treated with iodine in light or exposed to bright light, a number of interconvertible isomers, the so-called neo compounds, are produced (42, 70, 111 to 115). On adsorption col-

umns, some of these neo isomers are adsorbed above the original *trans* pigments, others are adsorbed below the *trans* isomers (42, 70, 115). These neo isomers contain *cis* configurations about some of the double bonds. When certain double bonds, indicated by *a* in the following partial carotenoid formula, occur with *cis* configuration, there may be slight steric interference between the methyl group and the hydrogen atom. Consequently, it is proposed that double bonds with this *cis* structure can not be present in carotenoid molecules (116, 117, 118).



On the basis of this prediction, the number of isomers formed by the action of isomerization catalysts must be much smaller (twenty for β-carotene) than would be expected if each of the double bonds was equally stable in the *cis* configuration (1,056 for β-carotene). As a rule, the number of neo isomers obtained by isomerization of the *trans* carotenoids is less than would be expected if each of the double bonds adjoining carbon atoms with attached methyl groups (bonds *b* in the formula) were equally stable in the *cis* configuration. From the ultraviolet absorption spectrum of the isomers and from a consideration of the probable stability of the bonds capable of existence with *cis* configuration, particularly the central, symmetrical double bond, spatial formulas have been assigned to many of the neo compounds (117).

Certain labile carotenoid pigments, such as prolycopene (116 to 123) which occurs in large quantities in fruits of the lily *Arum orientale* (56), are rapidly and completely convertible into the *trans* and neo compounds by the action of isomerization catalysts. On the basis of the prediction that certain double bonds can not exist with *cis* configuration, and since the absorption maxima of these labile pigments occur at wave lengths shorter than those of the *trans* pigments, it has been assumed that most of the double bonds adjoining carbon atoms with methyl groups occur with *cis* configuration (117). This assumption is based on the tenuous prediction that the thermodynamically least stable *cis* double bonds must possess so small an energy of ac-

tivation for transition to the *trans* form that the *cis* form is unstable at room temperature.

Molecular models of the carotenoids indicate that these pigments with all double bonds in *cis* configuration might be capable of existence in the protoplasm of specialized organs of plants. Those double bonds with very small energy of activation for spatial rearrangement would be isomerized during extraction of the pigments from the living organism. Molecular models also indicate that carotenoid molecules containing many *cis* double bonds would be shorter than those containing most double bonds in *trans* configuration; hence, x-ray analysis of crystals of the natural labile isomers might give definitive evidence regarding their spatial structure.

Pigments with different numbers of oxygen atoms but with the same polyene systems (β-carotene, cryptoxanthin, and zeaxanthin) yield different numbers of neo isomers; therefore, the hydroxyl groups must have an effect on the relative stabilities of the spatial isomers. Whether or not the isomerization phenomena play a role in the photochemical reactions involving storage of energy in green plants has not yet been demonstrated.

Adsorbability.—Of all the methods available for the separation and identification of chemical compounds, no technique has found such extensive use and has proved so efficient and reliable for investigation of the chloroplast pigments as chromatographic adsorption analysis. Indeed, much recent knowledge of the chlorophylls and carotenoids has been gained through application of this method. Use of the Tswett adsorption columns has made possible isolation of the pigments from extracts of plants, comparison of the pigments from various sources, comparison of the numerous artificial isomers with the native pigments, and quantitative estimation of the pigments (124 to 130). Nomenclature of the pigment isomers (70) and conclusions regarding their molecular structures (28) have been based upon their relative adsorbabilities. Experience gained in studies of the adsorbabilities of the leaf pigments has, in turn, aided improvement and applicability of chromatographic adsorption analysis.

In order to use the sequence of adsorbed pigments as a basis for nomenclature, for identification of pigments, and for estimation of molecular structure, particular attention must be given to the conditions under which the compounds are adsorbed. Relative positions of the adsorbed pigments may vary with changes of the solvent, of the adsorbent, or of both solvent and adsorbent (28, 51, 131). With mag-

nesia as adsorbent and with 1,2-dichloroethane as solvent, lutein is adsorbed below violaxanthin; but with a mixture of petroleum ether and acetone as solvent, violaxanthin is adsorbed below lutein. Solvents that reduce the adsorbability of chloroplast pigments usually decrease the sharpness of the spectral absorption bands.

Relative to the pheophytins, the chlorophylls are very strongly adsorbed; hence, the presence of the magnesium atom accounts for the greater adsorbability (and probably the greater polarity) of the green pigments just as hydroxyl groups account for the greater polarity of the xanthophylls relative to that of the similar, hydroxyl-free carotenes. Carotenoid pigments containing carbonyl groups usually exhibit considerable spectral change upon adsorption either at a solid-liquid interface or at a liquid-liquid interface (132, 133). This effect is particularly striking with fucoxanthin and peridinin. These adsorption phenomena may very well determine the condition and activity of the pigments within the chloroplasts. They may account for the marked increase in greenness observed when plants containing ketocarotenoids are killed with heat.

Photochemical and induced enzymatic oxidation.—Every farmer has observed the photochemical decolorization of the chloroplast pigments. If freshly cut grass or clover or alfalfa is cured in the shade or in large stacks, a deep green hay results; but if some of the same fresh material is cured in the sun, a pale, pigment-free hay is obtained. This bleaching effect of sunlight has been observed in many plants (134).

Preferential oxidation of the carotenoids upon exposure to sunlight of solutions containing these pigments and chlorophylls, observed by Noack nearly twenty years ago, necessitates the protection of plant extracts from strong light in the course of carotene analyses (43, 135). This preferential oxidation of carotene, which suggests that the chloroplast pigments may also exhibit different reactivities in the plant, is probably related to the action of the pigments upon other induced oxidations (136, 137). In the living plant, photoxidation processes occur even under natural conditions (138, 139).

Injury of plant tissues under conditions that are undestructive to enzymes usually results in a rapid oxidation of the chloroplast pigments. In the seeds of legumes this oxidative decolorization of the chloroplast pigments results from concomitant oxidation of unsaturated fats by a highly specific enzyme, a so-called unsaturated fat oxidase or lipid oxidase once called erroneously carotene oxidase (140,

141, 142). In leaves, especially in yellow seedlings grown in the dark, rapid oxidation of the carotenoids also occurs upon injury to the cells (42), but, according to unpublished investigations, this oxidation is not coupled with that of fats as it has been impossible to detect a fat oxidation catalyst. All these induced oxidations are inhibited by antioxidants (42, 43, 140 to 143).

Optical properties.—Both practical and theoretical problems hinge upon the optical properties of the chloroplast pigments. Absorption spectra serve as analytical standards, as criteria for purity of the pigment preparations, and as a basis for interpretation of the physical state of the pigments in the chloroplast. Knowledge of the spectral absorption characteristics of the pigments in solution makes possible estimation of the amount of light absorbed by each pigment in the plant.

Absorption spectra of the carotenoids (42, 114, 118, 144), of the chlorophylls (54, 55, 145 to 148), and of phycocyanin (14) determined in different laboratories are in very good agreement. Attempts to relate the wave lengths of the spectral absorption maxima of the chlorophylls to the refractive index of the solvent (Kundt's rule) have not been very successful except for solvents of similar molecular structure (148). Variation from the rule may be due, in part, to the formation of complexes between the pigments and the solvent. These variations are most apparent when pigments containing carbonyl groups in conjugation with the system of alternate single and double bonds are dissolved in polar solvents such as alcohols or ketones. Chlorophyll *b* with two carbonyl groups in conjugation with the same system of alternate single and double bonds found in chlorophyll *a* has its absorption maxima at much longer wave lengths when in solution in methanol than when in solution in nonpolar solvents. By contrast, chlorophyll *a* with only one carbonyl group exhibits more similar spectral absorption properties when dissolved in polar and in nonpolar solvents. Dissolved in nonpolar solvents, chlorophylls exhibit greater absorption coefficients than when dissolved in polar solvents.

Similar phenomena are observed with xanthophylls which contain carbonyl groups in conjugation with the polyene system. In general, such xanthophylls exhibit more pronounced absorption maxima when dissolved in nonpolar solvents than when dissolved in polar solvents. These effects may be made the basis for the prediction of the structure of xanthophylls. As an example, solutions of peridinin in petroleum ether exhibit several pronounced spectral absorption

bands; but solutions of this pigment in ethanol, in acetone, or in petroleum ether containing a few per cent ethanol exhibit a single broad absorption band; hence, the peridinin molecule probably contains one or more carbonyl groups in conjugation with the polyene system.

Absorption Spectra of Leaves

Even when computed on a comparable basis, absorption spectra of the green parts of plants do not as a rule correspond very well with the spectra of solutions of the pigments from the same plant material (14, 15, 82, 139, 149, 150, 151). Usually the absorption by the plant is too great in the regions where the extracted pigments absorb little light and too small in the regions where these extracted pigments exhibit maximum absorption. There are at least three principal conditions contributing to this disagreement. Pigments in the leaf occur in concentrations several thousand times greater than that of the pigments in the extracts utilized for spectral absorption measurements. For this reason, specific absorption values of the pigments in the two states may not be comparable. Scattering of light by colloidal material in the plant tends to increase apparent absorption values disproportionately in the spectral regions where absorption by the pigments is small. Owing to the concentration of pigments in discrete plastids, some light may pass through the plant without passing through the pigments, an effect that would cause the absorption by the plant to be relatively much less in the regions where the absorption by the pigments is greatest. In addition, the absorption maxima of chlorophyll in the leaf occur at considerably longer wave lengths than do those of chlorophyll in any known solvent. Whether this is due solely to the concentration of the chlorophyll, to its colloidal condition or physical state, or to other factors is not yet clear (28). Absorption spectra of pigments extracted from ruptured cells with aqueous solutions resemble spectra of living cells (151).

Utilization of Energy Absorbed by Various Pigments

Comparison of the amount of radiant energy utilized by the plant relative to that absorbed by each of the pigments has revealed that in diatoms some of the energy absorbed by carotenoid pigments (fucoxanthin) must be utilized in the photosynthetic reactions (152). In *Chlorella* and in *Chroococcus* (a blue-green alga) light absorbed by carotenoids is probably utilized but not so efficiently as that absorbed

by chlorophyll (14, 15). Light absorbed by phycocyanin in *Chroococcus* is utilized quite as efficiently as that absorbed by chlorophyll *a* (14), in spite of the fact that phycocyanin does not appear to be bound to the other pigments (28).

For plants such as diatoms, dinoflagellates, and brown algae that thrive at appreciable depths in the sea (20, 71, 72), utilization of energy absorbed by pigments other than chlorophyll *a* may be of especial importance. Because of the opacity of the sea to red and blue light, the submerged plants receive a preponderance of green light (3). Relatively little of this light is absorbed by chlorophyll *a*, whereas much of it is absorbed by the carotenoid pigments and by chlorophyll *c* (59, 152).

In diatoms the quantum yield of chlorophyll fluorescence is constant even when the exciting light is of such wave length that a large proportion is absorbed by carotenoid pigments. In acetone extracts of the diatoms, energy absorbed by the yellow pigments does not appear as chlorophyll fluorescence. The living organism must, therefore, contain a mechanism for the transfer of energy among the pigments (153). In all plants, the yield of chlorophyll fluorescence is small (154, 155). Extension of these measurements to plants containing bacteriochlorophyll and to those containing chlorophyll *d* may yield valuable information regarding the mechanism of energy transfer in the photosynthetic reaction.

Studies of the efficiency of photosynthesis (156) and of the mechanism of carbon dioxide reduction in nonphotochemical systems (157) have indicated that the photochemical reaction may consist of steps each of which need not contribute more than a small fraction of the amount of energy required to reduce one molecule of carbon dioxide. Further investigations may be expected to yield information about the mechanism of energy transfer from pigments to carbon compounds (153, 154, 155) and about the nature of the carbon compounds produced at this step (158).

LITERATURE CITED

1. Frolich, P. K., *Ind. Eng. Chem.*, **35**, 1131–38 (1943)
2. Riley, G. A., *Bull. Bingham Oceanog. Collection*, **7** (3), 1–89; **7** (4), 1–73 (1941)
3. Sverdrup, H. U., Johnson, M. W., and Fleming, R. H., *The oceans. Their physics, chemistry and general biology* (Prentice-Hall, Inc., New York, 1942)
4. Snieszko, S. F., *A symposium on hydrobiology*, pp. 227–40 (The University of Wisconsin Press, Madison, 1941)
5. Deevey, E. S., Jr., *A symposium on hydrobiology*, pp. 399–400 (The University of Wisconsin Press, Madison, 1941)
6. Burr, G. O., *A symposium on hydrobiology*, pp. 163–81 (The University of Wisconsin Press, Madison, 1941)
7. Handke, H. H., *Botan. Arch.*, **42**, 149–200 (1941)
8. Graham, H. W., *Sears Foundation: J. Marine Research*, **5** (2), 153–60 (1943)
9. Manning, W. M., and Juday, R. E., *Trans. Wisconsin Acad. Sci.*, **33**, 363–93 (1941)
10. Juday, C., *Trans. Wisconsin Acad. Sci.*, **34**, 103–35 (1942)
11. Worthington, E. B., *Nature*, **151**, 353–55 (1943)
12. Anonymous, *Nature*, **152**, 57–59 (1943)
13. Taylor, A. E., *Sci. Monthly*, 385–97 (1943)
14. Emerson, R., and Lewis, C. M., *J. Gen. Physiol.*, **25**, 579–95 (1942)
15. Emerson, R., and Lewis, C. M., *Am. J. Botany*, **30**, 165–78 (1943)
16. Spoehr, H. A., *Plant Physiol.*, **17**, 397–410 (1942)
17. Hartt, C. E., *Hawaiian Planters' Record*, **47**, 113–32 (1943)
18. Ivanov, L. A., *Chem. Abstracts*, **37**, 6703 (1943)
19. Frenkel, A. W., *Plant Physiol.*, **16**, 654–55 (1941)
20. Smith, G. M., *Cryptogamic botany* (McGraw-Hill Book Co., Inc., New York, 1938)
21. Roberts, E., *Am. J. Botany*, **29**, 16s (1942); Private publication (1943)
22. Inman, O. L., *Plant Physiol.*, **13**, 859–62 (1938)
23. French, C. S., *J. Gen. Physiol.*, **23**, 469–81 (1940)
24. Hill, R., *Proc. Roy. Soc.* (*London*) *B*, **127**, 192–210 (1939)
25. Hill, R., and Scarisbrick, R., *Proc. Roy. Soc.* (*London*) *B*, **129**, 238–55 (1940)
26. French, C. S., Newcomb, E., and Anson, M. L., *Am. J. Botany*, **29**, 8s (1942)
27. Boïchenko, E. A., *Chem. Abstracts*, **37**, 6005 (1943)
28. Strain, H. H., *J. Phys. Chem.*, **46**, 1151–61 (1942)
29. Smith, E. L., *J. Gen. Physiol.*, **24**, 565–82, 583–96 (1941)
30. Smith, E. L., and Pickels, E. G., *J. Gen. Physiol.*, **24**, 753–64 (1941)
31. Seybold, A., and Egle, K., *Botan. Arch.*, **41**, 578–603 (1940)
32. Hanson, E. A., Barrien, B. S., and Wood, J. G., *Australian J. Exptl. Biol. Med. Sci.*, **19**, 231–34 (1941)
33. Stoll, A., Widemann, E., and Rüegger, A., *Verhandl. Schweitz. Naturf. Ges.* (*Basel*), 125–26 (1942)
34. Galston, A., *Trans. Illinois State Acad. Sci.*, **35**, 66–67 (1942)

35. STRAUS, W., *Helv. Chim. Acta,* **25**, 179–88, 489–97, 705–17 (1942)
36. SINGH, B. N., AND RAO, N. K. A., *Current Sci.,* **11**, 442–43 (1942)
37. MOYER, L. S., AND FISHMAN, M. M., *Botan. Gaz.,* **104**, 449–54 (1943)
38. SOTO, V. S., *Bol. Biol. Univ. Puebla,* **1**, 9–17 (1942)
39. TIMM, E., *Z. Botan.,* **38**, 1–25 (1942)
40. MENKE, W., AND JACOB, E., *Z. physiol. Chem.,* **272**, 227–31 (1942)
41. MAYER, F., *The chemistry of natural coloring matters.* English translation by A. H. Cook (Reinhold Publishing Corp., New York, 1943)
42. STRAIN, H. H., "Leaf xanthophylls," *Carnegie Inst. Wash. Pub.,* No. **490** (1938)
43. PEPKOWITZ, L. P., *J. Biol. Chem.,* **149**, 465–71 (1943)
44. BUVAT, R., *Compt. rend.,* **213**, 660–63 (1941)
45. SEYBOLD, A., *Scientia,* **71**, 19–23 (1942)
46. SEYBOLD, A., *Botan. Arch.,* **42**, 254–88 (1941)
47. LINCOLN, R. E., ZSCHEILE, F. P., PORTER, J. W., KOHLER, G. W., AND CALDWELL, R. M., *Botan. Gaz.,* **105**, 113–15 (1943)
48. ZSCHEILE, F. P., BEADLE, B. W., AND KRAYBILL, H. R., *Food Research,* **8**, 299–313 (1943), and included refs.
49. SONNEBORN, T. M., *Cold Spring Harbor Symposia Quant. Biol.,* **10**, 111–24 (1942)
50. GRANIT, R., *Nature,* **151**, 631–32 (1943), and included refs.
51. STRAIN, H. H., *Chromatographic adsorption analysis* (Interscience Publishers, Inc., New York, 1942)
52. STRAIN, H. H., *Ind. Eng. Chem., Anal. Ed.,* **14**, 245–49 (1942)
53. ZECHMEISTER, L., AND CHOLNOKY, L. v., *Die chromatographische Adsorptionsmethode, Grundlagen, Methodik, Anwendungen. 2nd. Ed.* (J. Springer, Berlin, 1938); English translation, *Principles and practice of chromatography* (Chapman and Hall, London, 1941)
54. ZSCHEILE, F. P., *Botan. Rev.,* **7**, 587–648 (1941)
55. COMAR, C. L., AND ZSCHEILE, F. P., *Plant Physiol.,* **17**, 198–209 (1942)
56. STRAIN, H. H., AND MANNING, W. M., *Carnegie Inst. Wash., Yr. Book,* **42**, 79–83, 86, 87 (1943)
57. STRAIN, H. H., AND MANNING, W. M., *J. Biol. Chem.,* **144**, 625–36 (1942)
58. STRAIN, H. H., AND MANNING, W. M., *J. Am. Chem. Soc.,* **65**, 2258–59 (1943)
59. STRAIN, H. H., MANNING, W. M., AND HARDIN, G., *J. Biol. Chem.,* **148**, 655–68 (1943)
60. VAN NIEL, C. B., *Advances in Enzymology,* **1**, 263–328 (1941)
61. PACE, N., *J. Biol. Chem.,* **140**, 483–89 (1941)
62. ZSCHEILE, F. P., *Botan. Gaz.,* **103**, 401–3 (1941)
63. HEILBRON, I. M., *Nature,* **149**, 398–400 (1942)
64. HEILBRON, I. M., *J. Chem. Soc.,* 79–89 (1942)
65. SEYBOLD, A., EGLE, K., AND HÜLSBRUCH, W., *Botan. Arch.,* **42**, 239–53 (1941)
66. MONTFORT, C., *Z. physik. Chem.,* **A186**, 57–93 (1940)
67. KYLIN, H., *Kgl. Fysiograf. Sällskap. Lund, Förh.,* **9**, 213–31 (1939)
68. FOX, D. L., *Chronica Botanica,* **7**, 196–98 (1942)
69. STRAIN, H. H., AND MANNING, W. M., *J. Am. Chem. Soc.,* **64**, 1235 (1942)
70. POLGÁR, A., AND ZECHMEISTER, L., *J. Am. Chem. Soc.,* **64**, 1856–61 (1942)

71. Graham, H. W., "Studies in the morphology, taxonomy, and ecology of the peridiniales," *Carnegie Inst. Wash. Pub.*, No. **542** (1942)
72. Graham, H. W., *Carnegie Inst. Wash. Pub.*, No. **555**, pp. 1–10 (1943)
73. Heilbron, I. M., Jackson, H., and Jones, R. N., *Biochem. J.*, **29**, 1384–88 (1935)
74. Heilbron, I. M., Parry, E. G., and Phipers, R. F., *Biochem. J.*, **29**, 1376–81 (1935)
75. Carter, P. W., Heilbron, I. M., and Lythgoe, B., *Proc. Roy. Soc.* (*London*) *B*, **128**, 82–109 (1939)
76. Hey, D., *Biochem. J.*, **31**, 532–34 (1937)
77. Tippo, O., *Chronica Botanica*, **7**, 203–6 (1942)
77a. Kemmerer, A. R., and Fraps, G. S., *Ind. Eng. Chem., Anal. Ed.*, **15**, 714–16 (1943)
78. Kylin, H., *Kgl. Fysiograf. Sällskap. Lund. Förh.*, **7**, 131–58 (1937)
79. Ullrich, H., *Ber. deut. botan. Ges.*, **60**, 152–78 (1942)
80. Lärz, H., *Flora*, **35**, 319–55 (1942)
81. Gessner, F., *Jahrb. wiss. Botan.*, **89**, 1–12 (1940)
82. Montfort, C., *Botan. Arch.*, **43**, 322–92 (1942)
83. Bode, O., *Jahrb. wiss. Botan.*, **89**, 208–44 (1940)
84. Wendel, K., *Z. ges. Naturw.*, **6**, 327–29 (1941)
85. Seybold, A., *Botan. Arch.*, **43**, 71–77 (1942)
86. Sargent, M. C., *Plant Physiol.*, **15**, 275–90 (1940)
87. Went, F. W., LeRosen, A. L., and Zechmeister, L., *Plant Physiol.*, **17**, 91–100 (1942)
88. Olsen, C., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **24**, 99–103 (1942)
89. Haag, E., *Bull. soc. botan. Genève*, **33**, 1–71 (1941)
90. Stegmann, G., *Z. Botan.*, **35**, 385–422 (1939–40)
91. Thomas, M. D., Hendricks, R. H., Collier, T. R., and Hill, G. R., *Plant Physiol.*, **18**, 345–71 (1943)
92. Hambidge, G., "Hunger signs in crops," A symposium, *Am. Soc. Agron.*, and *Natl. Fertilizer Assoc.* (Washington, D.C., 1941)
93. Aronoff, S., and Mackinney, G., *Plant Physiol.*, **18**, 713–14 (1943)
94. Roberts, R. H., *Science*, **98**, 265 (1943)
95. Clausen, J., Keck, D. D., and Hiesey, W. M., "Experimental studies on the nature of species," *Carnegie Inst. Wash. Pub.*, No. **520** (1940)
96. Nakamura, H., *Japan. J. Botany*, **11**, 221–36 (1941)
97. Fischer, H., and Stern, A., *Die Chemie des Pyrrols*, II Bd., 2 Hälfte (Akad. Verlagsgesell., Leipzig, 1940)
98. Fischer, H., *Naturwissenschaften*, **28**, 401–5 (1940)
99. Godnev, T. N., *Chem. Abstracts*, **37**, 6703 (1943)
100. Mackinney, G., *Ann. Rev. Biochem.*, **9**, 459–90 (1940)
101. Mittenzwei, H., *Z. physiol. Chem.*, **275**, 93–121 (1942)
102. Franck, J., and Herzfeld, K. F., *J. Phys. Chem.*, **45**, 978–1025 (1941)
103. Franck, J., and Gaffron, H., *Advances in Enzymology*, **1**, 200–62 (1941)
104. Johnston, E. S., and Myers, J. E., *Ann. Rev. Biochem.*, **12**, 473–92 (1943)
105. Strain, H. H., and Manning, W. M., *J. Biol. Chem.*, **146**, 275–76 (1942)
106. Ruben, S., Frenkel, A. W., and Kamen, M. D., *J. Phys. Chem.*, **46**, 710–14 (1942)

107. Mackinney, G., and Joslyn, M. A., *J. Am. Chem. Soc.*, **63**, 2530–31 (1941)
108. Norris, T. H., Ruben, S., and Allen, M. B., *J. Am. Chem. Soc.*, **64**, 3037–40 (1942)
109. Manning, W. M., and Strain, H. H., *J. Biol. Chem.*, **151**, 1–19 (1943)
110. Mackinney, G., and Weast, C. A., *J. Biol. Chem.*, **133**, 551–58 (1940)
111. Zechmeister, L., and Tuzson, P., *Ber. deut. chem. Ges.*, **72**, 1340–46 (1939)
112. Zechmeister, L., Cholnoky, L. v., and Polgár, A., *Ber. deut. chem. Ges.*, **72**, 1678–85, 2039–40 (1939)
113. Zechmeister, L., and Escue, R. B., *Science*, **96**, 229–30 (1942)
114. Beadle, B. W., and Zscheile, F. P., *J. Biol. Chem.*, **144**, 21–33 (1942)
115. Strain, H. H., *J. Am. Chem. Soc.*, **63**, 3448–52 (1941)
116. Zechmeister, L., LeRosen, A. L., Went, F. W., and Pauling, L., *Proc. Natl. Acad. Sci. U.S.*, **27**, 468–74 (1941)
117. Zechmeister, L., LeRosen, A. L., Schroeder, W. A., Polgár, A., and Pauling, L., *J. Am. Chem. Soc.*, **65**, 1940–51 (1943)
118. Zechmeister, L., and Polgár, A., *J. Am. Chem. Soc.*, **65**, 1522–28 (1943)
119. Zechmeister, L., and Schroeder, W. A., *J. Am. Chem. Soc.*, **65**, 1535–40 (1943)
120. Zechmeister, L., and Escue, R. B., *J. Biol. Chem.*, **144**, 321–23 (1942)
121. Zechmeister, L., and Schroeder, W. A., *J. Biol. Chem.*, **144**, 315–20 (1942)
122. LeRosen, A. L., and Zechmeister, L., *J. Am. Chem. Soc.*, **64**, 1075–79 (1942)
123. Schroeder, W. A., *J. Am. Chem. Soc.*, **64**, 2510 (1942)
124. Wall, M. E., and Kelley, E. G., *Ind. Eng. Chem., Anal. Ed.*, **15**, 18–20 (1943)
125. Moore, L. A., and Ely, R., *Ind. Eng. Chem., Anal. Ed.*, **13**, 600–1 (1941); **14**, 707–8 (1942)
126. Peterson, W. J., *Ind. Eng. Chem., Anal. Ed.*, **13**, 212–16 (1941)
127. Rege, N. D., and Devadatta, S. C., *Current Sci.*, **12**, 21 (1943)
128. Zimmerman, W. I., Tressler, D. K., and Maynard, L. A., *Food Research*, **6**, 57–68 (1941)
129. Dutton, H. J., Bailey, G. F., and Kohake, E., *Ind. Eng. Chem.*, **35**, 1173–77 (1943)
130. Haagen-Smit, A. J., Jeffreys, C. E. P., and Kirchner, J. G., *Ind. Eng. Chem., Anal. Ed.*, **15**, 179–80 (1943)
131. LeRosen, A. L., *J. Am. Chem. Soc.*, **64**, 1905–7 (1942)
132. Danielli, J. F., and Fox, D. L., *Biochem. J.*, **35**, 1388–95 (1941)
133. Strauss, W., *Inaugural Dissertation* (Zürich, 1939)
134. Funk, G., *Ber. deut. botan. Ges.*, **57**, 404–13 (1939)
135. Aronoff, S., and Mackinney, G., *J. Am. Chem. Soc.*, **65**, 956–58 (1943)
136. Morgal, P. W., Byers, L. W., and Miller, E. J., *Ind. Eng. Chem.*, **35**, 794–97 (1943)
137. Jamieson, G. S., *Vegetable fats and oils* (Reinhold Publishing Corp., New York, 1943)
138. Franck, J., and French, C. S., *J. Gen. Physiol.*, **25**, 309–24 (1941)
139. Montfort, C., and Zöllner, G., *Botan. Arch.*, **43**, 393–460 (1942)

140. Balls, A. K., Axelrod, B., and Kies, M. W., *J. Biol. Chem.*, **149**, 491–504 (1943), and included refs.
141. Van Fleet, D. S., *J. Am. Chem. Soc.*, **65**, 740 (1943)
142. Van Fleet, D. S., *Am. J. Botany*, **30**, 678–85 (1943)
143. Williams, K. T., Bickoff, E., and Van Sandt, W., *Science*, **97**, 96–98 (1943)
144. Zscheile, F. P., White, J. W., Jr., Beadle, B. W., and Roach, J. R., *Plant Physiol.*, **17**, 331–46 (1942)
145. Zscheile, F. P., and Comar, C. L., *Botan. Gaz.*, **102**, 463–81 (1941)
146. Zscheile, F. P., Comar, C. L., and Mackinney, G., *Plant Physiol.*, **17**, 666–70 (1942)
147. Mackinney, G., *J. Biol. Chem.*, **132**, 91–109 (1940)
148. Harris, D. G., and Zscheile, F. P., *Botan. Gaz.*, **104**, 515–27 (1943)
149. Seybold, A., and Weissweiler, A., *Botan. Arch.*, **43**, 252–90 (1942)
150. French, C. S., *Botan. Gaz.*, **102**, 406–9 (1940)
151. French, C. S., *J. Gen. Physiol.*, **23**, 483–94 (1940)
152. Dutton, H. J., and Manning, W. M., *Am. J. Botany*, **28**, 516–26 (1941)
153. Dutton, H. J., Manning, W. M., and Duggar, B. M., *J. Phys. Chem.*, **47**, 308–13 (1943)
154. Franck, J., French, C. S., and Puck, T. T., *J. Phys. Chem.*, **45**, 1268–1300 (1941)
155. Kautsky, H., and Franck, U., *Biochem. Z.*, **315**, 139–232 (1943)
156. Emerson, R., and Lewis, C. M., *Am. J. Botany*, **28**, 789–804 (1941)
157. van Niel, C. B., *Physiol. Revs.*, **23**, 338–54 (1943)
158. Fan, C. S., Stauffer, J. F., and Umbreit, W. W., *J. Gen. Physiol.*, **27**, 15–28 (1943)

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MINERAL NUTRITION OF PLANTS

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Owing to conditions imposed by the war, the sources of literature covered by the present review are inevitably restricted. Nevertheless, of the available subject matter it is proposed to deal at some length with one or two aspects only of mineral nutrition, and to confine attention to the so-called major elements. Matters to be discussed in some detail are the relationship of potassium to other cations, and certain principles relating to the design and interpretation of complex experiments.

Absorption of ions, etc.—The old problem of the dependence on the transpiration stream of salt absorption and distribution has been re-examined by Broyer & Hoagland (1) in experiments which once more emphasise the importance of the metabolic status of the material used in studies of this kind. The absorption rate of young barley plants with a low-salt, high-sugar status was found to be closely dependent on such root factors as aeration and temperature, but largely unaffected by variations in transpiration rate brought about by differences in humidity and light conditions; absorption by roots higher in salt and lower in sugar, on the contrary, was appreciably affected by transpiration rate. The following explanation is advanced to account for this difference: increased growth and photosynthesis in the latter plant type under the favourable aerial conditions increased absorptive capacity through enhanced metabolic activity, while the capacity of the low-salt, high-sugar type was initially so great as to be affected relatively little by environmental conditions around the shoot, the duration of the experiment being limited. Reduced transpiration in some circumstances retarded or even prevented for a short period the movement of salts to the upper regions of the plant.

A contribution towards understanding the physiological basis of mutualism has been made by Routien & Dawson (2), in a study of the effects of mycorrhizal development on salt absorption, respiration rate, and growth in *Pinus echinata.* Using prepared clays with known exchange capacities, it was found that calcium, magnesium,

potassium, and iron were absorbed in much greater quantities, at low levels of base saturation of the clay, by the mycorrhizal than by the non-mycorrhizal plants; but at high degrees of saturation differences due to the fungus were slight. Respiration rate of unit length of root was increased two to four times by infection, and even greater differences were found in the anaerobic production of carbon dioxide. In view of the increased respiration it is suggested that "possession of mycorrhizae enables the plant to excrete more hydrogen ions for use in base exchange reactions with the soil colloids." While absorption of iron is regarded as one of the major roles of mycorrhizae in the growth and survival of *Pinus,* enhanced phosphorus uptake, claimed by earlier workers, was not confirmed.

The technique of supplying experimental plants with the bases in adsorbed form, as in Routien & Dawson's work (2), continues to attract more exponents and some of the work to be reviewed later employs such methods. A technique for nutrition studies has been outlined by Converse *et al.* (3), who claim by its use growth responses and deficiency symptoms more definite than with solution cultures. Two other papers on this subject are of importance: Coleman (4) showed that oats and cotton can utilise in large amounts clay-adsorbed phosphate which is not removed by extractants commonly employed in the determination of readily available phosphate in soils, while Graham & Albrecht (5) demonstrated that adsorbed nitrate is available to plants as readily as are adsorbed cations.

Experiments of considerable interest, relating to the mechanism of auxin action, have been described by Commoner *et al.* (6). Thin discs of potato immersed in hypertonic sucrose solutions lost water during six days, but with addition of 10 mg. of indole-3-acetic acid per liter only a slight initial loss occurred, with subsequent full recovery; whereas when potassium chloride or fumarate was also added water was actually taken up. Since auxin can bring about absorption of water in flaccid cells against an osmotic gradient, restoring turgidity or even resulting in cell enlargement, the theory is disproved that it acts primarily on the cell wall. It is argued that the considerable water absorption from an external solution of salts suggests that auxin may act by increasing the amount of solutes in the cells. This argument is perhaps not so convincing, but further work (7) showed that auxin affected salt uptake by potato discs in a manner parallel to its influence on cell enlargement. From these and other experiments

it is concluded that the effect of auxin on cell enlargement depends upon its effect on salt absorption.

The dependence of cell enlargement on respiratory processes may be viewed as a consequence of the respiratory dependence of the salt absorption process, and the evidence points to the four-carbon acid metabolism as the respiratory agent particularly related to these phenomena.

Remarkable results relating to the problem of chlorosis in water culture have been reported by Went (8), which, if substantiated, will have important bearings both on theoretical physiology and on the methods of water culture. According to Went, the chlorosis and poor top growth often obtained in solution cultures cannot be prevented by aeration, although they may be avoided by careful control of the pH and by raising the iron level to many times that required in sub-irrigation techniques. If, on the other hand, the root system be divided, one portion remaining in the solution while the other is allowed to develop in moist air, even at low iron levels the chlorotic symptoms disappear and growth of the tops becomes maximal. A very small proportion of the root system outside the solution may perform this function; indeed, even roots developing in the saturated atmosphere above the solution may be effective. Internal secretion by the aerial roots of "caulocaline," a specific factor for shoot growth, is postulated to explain the phenomenon. Clearly, plants differ much as regards the aeration necessary for their roots, some apparently being independent of it. Went, however, considers that in general plant roots exhibit a division of labour, deep roots being concerned with the absorption of water and salts, and surface roots with caulocaline production. It is difficult to envisage the respects in which roots growing in moist air, and surrounded by a water film, differ from those growing in efficiently aerated solutions, as regards either access to oxygen or freedom from carbon dioxide accumulation. It would appear therefore that if aerial roots really act in this way, caulocaline production must either be inhibited by the presence of salts (in which case transference of the roots to pure water should enable it to proceed), or else the hormone or some precursor must escape from submerged roots.

Diagnosis and assessment of deficiency.—In agricultural and horticultural practice early diagnosis of unbalanced nutrient conditions is always an urgent need, and one to which increasing attention is being devoted. Wallace has specialised for many years in diagnosis by

visual inspection alone, and has now published a series of attractive colour photographs (9). While these should be of much value to field workers, much experience will be required to train the eye sufficiently to discriminate definitely between more than a few of the better-defined symptoms. Diagnostic methods involving analytical or plant-tissue tests have been discussed recently in several papers (10 to 13). Methods suited to pineapples have been elaborated and illustrated by Nightingale (14, 15) in two papers meriting attention. The first paper has already been reviewed (16), and the main principles outlined; in the second, inter-relations of the three major nutrient elements are considered in some detail. Although fruits from plants subjected to variations in phosphorus and potassium supply differed much in size, they were found to be similar in flesh colour and texture, sugar content, acidity, etc. This is attributed largely to an interplay of compensating relationships, e.g., when phosphorus and potassium are both low, carbohydrate accumulation is also curtailed; the low potassium level limits nitrate absorption, while reduced nitrogen in its turn allows freer absorption of the available phosphate. The net result is a condition of "proximately balanced multiple deficiencies," reflected in reduced size largely unaccompanied by other adverse effects. The subject is again discussed in another paper (17). While recognising as a general principle the capacity of plants, within wide limits of available nutrients, for adjusting uptake and utilisation to secure high efficiency, yet in this instance the explanation offered may not be entirely cogent; for Arnon & Hoagland (18), working with tomatoes, have demonstrated that over a wide range of potassium and phosphorus nutrition the mineral composition of the fruit is remarkably constant, in strong contrast to that of the vegetative parts. Similar constancy was found whether the plants were grown in soil, water, or sand, and over a three-fold concentration range of the culture solution.

Olsen & Shaw (19) investigated in six Ohio soils the relation between crop response to potash fertilisation and available potassium. In order to evaluate the latter, chemical and biological (Mitscherlich and Neubauer) tests were run concurrently with the field trials. All methods agreed in ranking the soils in the same order as did the field responses, the biological methods giving rather better differentiation than the chemical tests. In the field trials each soil was investigated at four potassium levels over a period of three years, the layout being in each case a Latin square; the results were examined using the analysis

of variance. These field results have been re-examined by Willcox (20) from the agrobiological point of view, and some of the data have been fitted into the "universal yield diagram." The claims for this method are far-reaching, and the underlying principle on which are based estimates of potash availability and general fertility level is not at fault, namely a comparison between the slope of the response curve and the absolute level of yield. A fundamental assumption as to the form of the response curve is made, however, which conflicts with the evidence in the experimental data; to be serviceable the "universal yield diagram" is entirely dependent upon the observed response curve conforming to the Mitscherlich type. Willcox rejects the practical relevancy of the analysis of variance, and appeals to the eye as a guide in fitting observed values, which show no statistical evidence of differences, on to the theoretical yield curves. Examination of the data leads irresistibly to the conclusion that the analysis of variance is in this instance a more reliable guide than Willcox admits.

Calcium/potassium ratio.—Interest in problems of potassium nutrition has characterised the year under review, owing largely to the publication of twelve addresses on the subject delivered to the American Chemical Society (21). The themes chiefly concerned are the factors affecting potassium absorption and the relation of other cations, particularly calcium and sodium, to potassium. Some of these questions will be examined in considerable detail, and in order to stress salient points full use will be made of two papers already briefly noticed in last year's review (16).

Since variation in the Ca/K ratio of soils is reflected within the plants naturally growing on those soils, the use of the ratio is suggested, not for the first time, by Albrecht (22) as a basis for an ecological classification. When the ratio is low, growth is increased, and both percentage and total contents of nitrogen and phosphorus are reduced: the crop is "carbonaceous." With high ratios the vegetation is more "proteinaceous." In this connection may be cited the work of Chu (23), who found that different species of planktonic algae have variable requirements in the way of calcium, magnesium, potassium, and sodium, but are much more constant as regards nitrogen and phosphorus requirements (the optimum levels of the latter are very low, for nitrogen 1 to 7 p.p.m., and for phosphorus 0.1 to 2.0 p.p.m.).

Peech & Bradfield (24) state that addition of lime to soils containing neutral salts either may not affect, may decrease, or may increase the concentration of potassium in the soil solution, according to the

initial degree of base saturation. They believe that calcium has little effect on the absorption of potassium, at least at concentrations such as occur in soil solutions, and conclude indeed that the apparently contradictory results concerning the calcium-potassium relationships in soils and plants are in fact in fairly good agreement. This view would seem probably to over-simplify the position, as appears from another discussion of the relevant data by Pierre & Bower (25). They point out that calcium may increase potassium uptake when the concentration of the latter is high and the Ca/K ratio relatively low, or when the concentrations of sodium and possibly other cations are high relative to potassium; but that where calcium is present in high concentration it reduces potassium absorption. They postulate that the effect of any cation on potassium absorption depends on (*a*) potash level, (*b*) relative concentration of the cation to potassium, (*c*) the specific cation, (*d*) presence of other cations, (*e*) plant species, and (*f*) pH. When to these factors are added others due to the kinds and amounts of the various anions, some notion is gained of the complexity of the situation. In agreement with Peech & Bradfield (24) they infer the probability that "ion competition" is more pronounced at high concentrations than at low, for a given ratio of the cations. Potassium has a higher "competitive ability" than other common cations, hence calcium and magnesium do not reduce potassium absorption to the same extent as does potassium that of the divalent elements.

Further studies of the Ca/K ratio in nutrition are found in other papers (26, 27), but the most revealing results are those of Hunter *et al.* (28). Prepared potassium-, calcium-, magnesium-, and phosphate-soils were mixed in suitable proportions to give exchangeable Ca/K equivalent ratios of 1, 2, 4, 8, 16, and 32 respectively; since the sum of the two cations studied was approximately constant, initially the soils contained very different absolute levels of these nutrients. Seven successive crops of alfalfa were grown on each mixture, gradually exhausting the soils, and from analysis of the crops it was possible to follow approximately the changing nutrient conditions for each. The important result was obtained that over a very wide range of Ca/K ratios (1 to 100) in the medium, the yield was almost uniformly good so long as the soil contained more than a critical limit of potassium. When the Ca/K equivalent ratio in the plant tops exceeded about 4.0, yield was depressed, but a differentiation just as good as regards response was given by the absolute contents of either calcium

or potassium alone; thus, whenever calcium in the tops exceeded 2 per cent, or potassium fell below 1 per cent, the yield dropped abruptly. This result throws much light on the value of the Ca/K ratio. Without doubt the dominant factor throughout the experiment was the absolute amount of potassium available to the plant; the response of alfalfa at least is determined primarily by the absolute internal level of potassium. Its relation to calcium is of quite secondary importance, for yield is not markedly reduced unless the supply of potassium is so inadequate that no adjustment of the other cations can compensate for the lack of it. The contents of other cations may have comparatively minor effects on that yield, but probably would be reflected mainly in the symptomatic response of the plant. The interpretation of all work relating to the relative concentrations of two or more nutrient elements must include assessment of the effects of the absolute as well as the relative contents, a consideration so obvious that it is sometimes apt to be overlooked.

The main requirement then is a knowledge of the effects on yield etc. of varying content of either element at each content level of the other, a relationship such as could easily be illustrated in a solid model, or by contour lines in a square diagram. Whether simpler prediction formulae for this yield surface could be obtained from (*a*) the sum of the concentrations, and (*b*) their ratio, rather than more directly from the individual concentrations themselves, is very doubtful; it is, however, certain that adequate prediction over the whole surface will not be obtained if the two degrees of freedom represented by the independent variables are artificially reduced to one, as happens when the ratio alone is considered. The point will be amplified later.

Potassium deficiency and accumulation of other ions.—This discussion has led to a consideration of the possibility that the diagnostic symptoms of potassium deficiency may differ according to the relative contents of cations other than potassium—nor need possible effects of anions and carbohydrates be excluded. It is characteristic of potassium deficiency that other elements accumulate in the cells; should these accumulations reach toxic levels, definite symptoms may be expected to arise, and there is no apparent reason why accumulations of different elements should not lead to the development of diverse symptoms. The variety of symptoms found in barley has in fact been attributed to such accumulations (29). Hence the investigations of Chapman & Brown (30), dealing with symptoms in citrus

under potassium deficiency, are of interest; solutions were used both high and low in calcium, magnesium, sodium, nitrate, sulphate, and chloride. Two effects were attributed by the investigators to toxic accumulations: a leaf burn due to sodium, and in lemon a leaf pattern typical of boron excess; boron was found to have accumulated to some six times the normal content in leaves so affected. Apart from these two effects the symptoms developed in the various solutions were generally similar, a fact which is interpreted as indicating that they were primary, being "associated with some fundamental disturbance of plant metabolism caused by potassium deficiency." This interpretation may be questioned.

Accumulations of calcium, magnesium, and nitrogen were encountered in the experiment; phosphorus, sulphur, and chlorine were nowhere absorbed in excess. Sodium accumulations are claimed, but actually the contents were always very low; only in one instance was so much as 0.5 per cent of the dry weight recorded, and even here the calcium content was more than five times as great as that of sodium, although in the solution sodium was present to five times the calcium level. Citrus is evidently incapable of absorbing large quantities of sodium. Even so, Chapman & Brown claim that these low sodium contents resulted in a leaf burn. Under the circumstances, the general constancy of the symptoms might well be attributed to the similarity in composition, and be due in large measure to effects of excess calcium and magnesium. In high-sodium nutrients the onset of adverse symptoms was delayed; here, while sodium itself was not absorbed excessively, calcium and magnesium accumulation was not as excessive as in other treatments, and was presumably retarded. In barley, composition is much more variable (31) and the variability is reflected in the symptoms accompanying deficiency. The conclusion that the symptoms described are primary in character is therefore open to serious question. Growth rate was apparently retarded long before other symptoms appeared, and this retardation might well be a primary effect; accumulations, resulting largely from this, reached toxic levels and characteristic abnormalities appeared.

The question of toxicity as related to potassium deficiency was also raised by Mullison & Mullison (32). Previous work (33) had provided evidence of toxic effects due to excess phosphorus under nitrogen deficiency, and had also shown that in a high phosphate solution, in the absence of sodium, symptoms of potassium deficiency were very severe, appearing in two or three days. The Ca/Mg ratio

in the solution made little difference to the severity of the disorder, though as the ratio became very small the symptoms changed. The later work demonstrated that in the presence of sodium, symptoms were much milder. The questions therefore were raised whether those symptoms were aggravated by phosphate toxicity, and whether sodium could largely replace potassium. To investigate these matters barley seedlings were grown in the same types of solution as before, but at differing phosphate levels, and over two pH ranges, both in the presence and absence of sodium. The lower pH range was sufficiently acid to be highly detrimental, and here there was evidence of phosphate toxicity. At the higher range, however, little evidence emerged of differential toxicity due to phosphate, but again sodium ameliorated the condition. Hence it was concluded that the severe dieback found in their experiments

must have been caused by the fact that there was no sodium present to substitute for the lack of potassium; [it was] due to a deficiency of potassium rather than to interrelations of PO_4 and NO_3, or Ca and Mg, or a toxicity of one of these.

As to phosphorus toxicity, the very high and excessive level of phosphorus nutrition should be stressed, both as absolute concentration and also relative to nitrogen, even in this lowest phosphorus treatment. There can be no doubt that at the lowest level, in the absence of potassium, internal phosphorus content was excessive and probably but little below that developed from the higher phosphorus solutions. Data have been presented (29) showing that in barley, under nutrient conditions closely similar to those described here, phosphorus accumulates internally and becomes highly detrimental even from much lower external concentrations than the lowest used by Mullison & Mullison. Hence there can be little doubt that toxicity due to this element was an important contributory factor in the deterioration of the plants. Nor does the writer see in the data and discussion presented any reason to doubt that calcium and magnesium accumulations occurred, just as in the experiments of Chapman & Brown (30); indeed in these latter data, for leaves of a given age, the highest content of calcium was found in plants supplied with a large excess of phosphorus.

The effect of sodium.—In order to investigate more fully the effect of sodium, Mullison & Mullison grew barley in a solution free from the element with concentrations of potassium ranging from 0 to 180 p.p.m., while in a parallel run the same treatments were applied except that the deficit of potassium below 180 p.p.m. in each solution

was made up with sodium. In this experiment considerable improvement in dry weight was again found, the result being interpreted as further evidence that "sodium itself is utilized in place of the potassium when the latter is present in insufficient amounts." In the writer's view this is a misconstruction, since the sodium effect was found even at high potassium levels, where the actual amount of sodium presented was smaller than at lower potassium levels. Clearly the improvement due to sodium at high potassium levels was something added to the potassium effect, and not a substitution effect, for potassium was already in a concentration high enough to give its maximum yield. The highest yields were given by concentrations of 20 to 40 p.p.m. of potassium combined with 160 to 140 p.p.m. of sodium, and in the absence of sodium the yield at all potassium levels was lower. There is no reason for assuming that at the lowest potassium levels the similar improvement in yield following sodium addition, even if relatively greater, was in any real sense a substitution effect. Indeed results obtained at this Institute indicate that the increased growth rate in barley, resulting from the addition of a moderate amount of sodium to a high calcium solution virtually without potassium, is by no means indicative of generally improved physiological conditions. Size of individual leaves, and total leaf area, are increased, but assimilation rate is reduced. Tillering does not stop at the normal time and becomes excessive; in spite of the increased tiller number practically no ears reach maturity, and no grain is produced. In the absence of sodium, on the contrary, half the ears are carried to maturity in the normal time and an appreciable yield of good grain results.

Analyses of these plants, grown in nutrient media designated as HCK_5 and HDK_5 (31), indicate that calcium uptake is reduced by sodium, which itself is readily absorbed, resulting in a high and probably detrimental sodium content; the effect on magnesium is unknown, but it is likely that accumulation of both divalent elements is reduced. It is possible that the increased vegetative growth following sodium addition is due rather to the reduction of calcium than to the presence of sodium. As in the experiments of Mullison & Mullison, the modification of plant type induced by sodium is found also at higher potassium levels, but with diminishing intensity. Reference may also be made to the phenomena found under acute potassium deficiency in the absence of sodium and the presence of only small amounts of calcium, when nitrate and phosphate are supplied as am-

monium salts (29). Here the symptoms appear as quickly and are quite as severe as those described by Mullison & Mullison (32); rapid death of foliage and plants leaves no doubt that poisoning is the main cause of deterioration. Addition of sodium, instead of improving matters, results here in more complete destruction; presumably there can be no excess accumulation of calcium in any event, and sodium cannot now result in improvement, though rubidium does so. It may also be noted that in these plants, in the absence of sodium, reduction of phosphorus supply diminishes the toxic symptoms, and if continued far enough eliminates them altogether.

Lehr (34) attacked the same problem from the standpoint of the relative concentrations of potassium, sodium, and calcium in the beet. Relative contents of the three cations for each treatment were plotted in triangular diagrams, leaves and roots being treated separately; in both triangles, regions of roughly equal yield could be delimited. From the observed distributions it was deduced that in the root, sodium is able very largely to substitute for potassium; it has moreover a second important function "as a stimulant to production." In the leaf the second effect is much more pronounced, but "it is probable that sodium does *not* play the part here of a replacement." Is then the function of potassium different in root and leaf?

According to Lehr

> the inclination has been too great to evaluate the importance of an element according to its absolute quantity. In the equilibrium of cations, however, the decisive factor is the exact proportions rather than the absolute quantities.

Neither aspect should be neglected, and Lehr's own interpretation suffers somewhat from neglect of the latter. Yield certainly depends on the absolute content of potassium and, over a narrower range, of calcium also; when potassium is low no adjustment between sodium and calcium can compensate for the lack. Thus by the use of ammonium salts to provide nitrogen and phosphorus, and a careful regulation of low external concentrations of the cations, yields might presumably be procured much below the lowest observed in this experiment, yet occupying the same region of the triangle as do actually the highest. Hence the topography of such a figure is fortuitous in the sense that it is largely determined by the particular combination of treatments from which it is derived. In order to avoid confusion with effects of absolute potassium content one of two conditions would need to be fulfilled. First, sufficiently high potassium levels could be used to ensure that its content variations would not in themselves

limit yield, a condition apparently nearly fulfilled in Lehr's experiment; it would then, however, be automatically impossible to obtain substitution effects of sodium. Second, by suitable adjustment of the culture solutions the potassium content of the plants might be regulated at a low but constant level, so that growth would be directly limited, while substitution effects, if any, might contribute to yield. Even under these hypothetical conditions it is not obvious what criteria could be used to decide whether an effect ascribed to sodium was in fact a substitution effect for potassium, or one not directly related to that element. It may be noted too that each yield position in the triangle could now be interpreted in two ways, either in relation to the proportions of the three variables, or to the absolute content of calcium and sodium. A more logical and comprehensive approach to the problem of the interrelations of the ions in growth is outlined in the final section of the review.

Lehr's data (34), consistently with many others on beet (35) and mangold,[1] etc., show an appreciable response to sodium. In its absence, at the low potassium level, calcium accumulated to very high concentrations in the leaves, but the content fell as potassium level increased. Sodium acted similarly on calcium absorption, and similarly increased yield, but produced a different type of plant. Presumably the high calcium content is again detrimental, and these particular crops may respond so well to sodium because of their inordinate ability to absorb calcium. Again, sodium may entail greater phosphate absorption (31, 37), while it is possible that internal excess calcium may in its turn interfere with phosphorus metabolism and partly account for the calcium type of plant "characterized chiefly by compressed habit and dark green colour of foliage." Assuming that the ratio of the total weight of the plant to that of the leaves provides a rough measure of assimilatory efficiency, in Lehr's experiment leaf size and efficiency were not coordinated, for high efficiency required higher potassium and lower sodium content than large leaf size. In the experiment, too, the highest sodium level was definitely detrimental. These effects all resemble those found in barley, and mentioned earlier, and there can be little doubt that in both plants the phenomena are of the same general type, differing only in degree.

In the writer's view, the most likely relationship of sodium to

[1] See an interesting survey by Watson & Russell (36) of the data obtained in the Barnfield experiment at Rothamsted over a period of more than sixty years.

potassium may be summarised thus: sodium cannot perform the primary essential function of potassium. When the potassium level is too low to exert this function adequately, toxic accumulations of other elements may occur, producing characteristic symptoms and reducing growth still further, or even leading to death of the plant. Sodium may hinder or entirely suppress such accumulations, thus improving the general condition; it may also increase phosphorus uptake, with either favourable or detrimental results. In some plants, e.g., barley, however, sodium may itself accumulate to toxic levels, resulting in secondary injury of a modified type; but in others (beet, mangold) higher internal concentrations are tolerated and considerable improvement results. In these plants sodium may exert a beneficial effect even when the potassium supply is sufficiently high not to limit growth directly; but in barley at high potassium levels large increases in yield due to sodium are not found, although the plant type is again modified in its presence. The toxic effects postulated may indeed be due to unbalanced values of the internal cation ratios, but probably are due largely to direct accumulations.

It is of considerable interest that in Lehr's experiment (34) potassium appeared in much lower concentration in the leaf than either sodium or calcium, while in the root it constituted the dominant element. A high potassium content in potato tubers, as contrasted with high calcium in the shoots, has likewise been recorded by Schroeder & Albrecht (26). A strikingly similar difference in the mineral composition of leaf and fruit in tomato has been noted by Arnon & Hoagland (18), and again in strawberry by Lineberry & Burkhart (38). The physiological similarity between these types of "storage organ" is thus emphasised. Closer enquiry into the causes and consequences of this differential cation distribution should be profitable.

Nutrient effects in metabolism.—A study of the onset of sulphur deficiency in *Chlorella,* and of recovery from it, has been presented by Mandels (39). As deficiency progresses, chlorophyll formation slows down and is superseded by decomposition and chlorosis; cell-division also stops, though chlorophyll formation decreases more rapidly. Chlorotic cells accumulate fat instead of starch. During recovery, chlorophyll formation proceeds exponentially and at a higher rate than in normal cells; it precedes cell-division and the final content is much above normal. About one mole of chlorophyll is formed for every three moles of sulphate supplied. Deficient *Chlorella* is able to utilise sulphur from many inorganic sources (hydrogen sulphide, thiosul-

phate, pyrosulphate, sulphite, persulphate), but from no organic compound so far tested. In this connection Thomas *et al.* (40), in a comprehensive study of sulphur nutrition in alfalfa, demonstrated that under deficiency the yield may be improved by fumigation with sulphur dioxide, though this is a less effective source than sulphate. Plants growing in the presence of sulphate absorb less from the solution if fumigated. Further work on sulphur metabolism has been reported by Eaton (41, 42) and a final survey of his series of investigations has also been presented (43). He concludes that in sulphur deficiency increased proteolysis probably occurs concurrently with reduced protein synthesis, accounting for increased soluble organic nitrogenous compounds. At the same time reductase activity is low, leading to nitrate accumulation, hence there is a "rather unique mechanism that enables the plant to carry on the processes of proteolysis and starch accumulation at the same time." The argument for increased proteolysis is based largely on a lowered reducing sugar content, that for retarded synthesis on a presumed deficiency of sulphur-containing amino acids. The position, however, would seem to be complex, since soluble organic sulphur actually accumulates.

Two further contributions have appeared from the Adelaide school (44, 45) on the inter-relations between respiration rate and protein, carbohydrate, and water contents. Using *Phalaris tuberosa*, changes in these characteristics in the leaves were determined during five days following differential treatment with sucrose and ammonium chloride. The view is maintained that proteins are synthesised directly from all the amino acids rather than along any alternative path, and in fact extremely good prediction of protein content could be obtained from amino acid and water contents considered together. Ammonia showed no relation to protein, while respiration rate was closely related to amino acids; it is suggested that the latter are oxidised differentially. This is not the place to enter into a close discussion of these matters; indeed, it would appear that experiments along these lines cannot supply final answers to the questions at issue. It may however be emphasised that the experiment in general constituted a study of net protein hydrolysis on a considerable scale and of export of nitrogen from the leaves to unspecified destinations. None of the treatments resulted in increased protein content, and sucrose addition was strikingly detrimental: on the fifth day the mean protein content of the leaves of the sucrose treated plants was 1.71 per cent, while prior to treatment it had been 2.72 per cent; this fall appar-

ently cannot be accounted for by sucrose uptake. Abnormal metabolism may therefore have been studied, and it is more than doubtful if the various correlations found are typical of those ordinarily obtaining. For example, in two treatments, while this breakdown was occurring, ammonia was being forced into the leaves; this might well obscure relationships to be expected on some theories between ammonia and protein in normal metabolism. The good prediction obtainable for protein from total amino nitrogen and water contents may arise very largely from causes other than that to which it is attributed by Wood & Petrie (44). The relationship with total amino nitrogen may be seen from a correlation diagram to depend almost entirely on the fact that plants from the two treatments given nitrogen in the absence of sugar behaved quite differently from those in the remaining five.[2] Instead of losing nitrogen, they maintained their protein while amino nitrogen, along with other soluble forms, increased as a result of the nitrogen supplied. Within neither group was there appreciable evidence of a simple relation between protein and amino nitrogen content, but the pair given nitrogen without sugar naturally contained much more nitrogen in all forms than the other five. Hence the observed total correlation depends very largely on the fact that the leaves in some treatments were losing nitrogen and in others gaining it. A clear discussion of the validity of correlation coefficients in data of this kind has been presented recently by Welker & Wynd (46).

Again, much of the correlation with water must certainly arise from hydration of the proteins themselves; hence the good prediction obtained for protein when water content is one of the independent variates gives little indication of the extent to which protein has in fact been determined by water content. The inverse relationship, the physiological dependence of water content on protein content, is implicit in the work of Mason & Phillis (47, 48, 49), who consider the primary determinant of water content to be a "bulk factor," i.e., the amount of anhydrous protoplasm. Variation in water content, when measured in terms of anhydrous protoplasm, is attributed to variation in hydration capacity, this being determined by the salt content. Osmotic vacuolar effects are of minor importance. This work is open to the criticism that salt content is estimated as the product of water content and electrical conductivity of the sap; hence correlations with water content are artificially exaggerated. The conclusion that their results demonstrate a close dependence of water content on salt con-

[2] See Fig. 2 in reference 45 for protein vs. residual amino nitrogen.

tent, with no evidence of differential effects of the various ions, rests therefore on insecure foundations. Not only this work, but other conclusions reported in the previous review (16) and relating to the disturbing effect of size of plant on water content in potassium deficiency, and to the theory of apolar adsorption in metabolism, have been radically criticised elsewhere by the present writer (50).

Triangular diagrams and experimental designs.—Increasing use is being made of the triangular diagram for representing the dependence of some measured characteristic, such as yield, on the relative content of three nutrient ions. As was seen earlier, the method has serious theoretical limitations, due very largely to the fact that in it two degrees of freedom only are used to evaluate results obtained from a physiological system involving three. This might be overcome by considering a series of triangles, within each of which a particular constant sum of the three ions under study is maintained. Such a set of figures would constitute cross-sections of either a triangular prism or pyramid, the solid enclosing contour shells. With sections graded in size to form a rectangular triangular pyramid, the solid would become simply the corner of a cube. Moreover, the three cube edges would now represent the axes of an orthogonal coordinate system, each axis corresponding to one degree of freedom and representing one of the three ions in isolation from the remaining two. This arrangement is the most rational geometrical representation of the interacting effects of three ions, and applies equally whether the ionic concentrations, whose effects are under investigation, are those within the plant or in the nutrient solution. Not only can triangular sections be derived from a cube, but sections parallel to one face give immediately contour maps of the interaction between two of the ions at constant levels of the third. Until data are forthcoming for the construction, even approximately, of such a solid, effects of the three ions are insufficiently known and still less understood; clearly it must be an objective of nutritional research to obtain the necessary data from representative plant types. The principle may of course be extended to include into one orthogonal scheme as many ions, both positive and negative, as necessary.

The three-salt technique extensively used in nutrition experiments has been developed along lines which allow a "triangular layout" such that the cations are virtually constant, while allowing full variation in the anions, or vice versa. The method was first described by Hamner (51) and has been used in several of the investigations

reported here and in the review of last year (32, 33, 52, 53). If three anions are combined factorially with three cations, nine salts are obtained: say the nitrates, phosphates, and sulphates of potassium, calcium, and magnesium. By combining the three nitrates, one of the solutions necessary for the "anion triangle" is obtained; the other two are similarly compounded from the phosphates and sulphates respectively. These solutions occupy the apices of the triangle and by suitably mixing them the remainder of the triangle may be laid out in the usual manner. By grouping and treating similarly the cations in the original salts a "cation triangle" is obtained over which the anions are constant. Clearly the method could be extended, and, for instance, from four anions and four cations two tetrahedra could be erected, all of whose sections would be anion or cation triangles.

The method is very elegant and attractive, but before being widely adopted its limitations should be considered and weighed against those of other possible arrangements, the most generally useful of which is undoubtedly the factorial. While the claim is readily admitted that in these triangular arrangements the only variable constituents of the solutions are those under study, yet in culture solutions no single ion can be varied alone, hence uncertainty must always exist as to the manner in which the resultant effects on the plant should be apportioned among these experimental factors. The very effects it is desired to measure are confounded, unless indeed the fallacious assumption is made that these effects are confined to the relative concentrations of the elements, a matter which has already received attention. This difficulty is met in all work involving the triangular design. In factorial experiments, on the other hand, an extra ion must be added to the system in varying quantity; there is here no confusion among the effects of the ions under investigation, though some may exist between these effects and that of the "balancing ion." A certain amount of latitude exists in the choice of this ion, however, and of the concentration range over which its variability falls; a judicious choice will usually ensure minimal confusion. With due regard to the limitations of the two methods the advantage undoubtedly lies with the factorial design.

Linked with the objections already raised to triangular arrangements is the overriding consideration that no statistical assessment is possible either of general effects of the ions under study or of their interaction effects with others. Moreover, the subdivision of the investigation into two independent parts, represented by the anion and

cation triangles, precludes any study of "interaction" phenomena between the anions and cations. The desirability of statistical estimation was appreciated by Hamner *et al.* (52), who treated one of their experiments as randomised blocks. An accurate estimate of error was available, and by its use differences were readily demonstrable among the eighty-six treatment degrees of freedom. But further analysis along these lines was impossible, and effects of the individual ions could be apportioned only by inspection. Concerning interactions, Lyon *et al.* (53) state:

The interaction of certain ions is obvious. Thus, at concentrations of calcium of 5.7 milliequivalents, greater fruitfulness resulted when potassium was relatively low and magnesium relatively high. Other examples could be cited . . . .

Interaction as generally understood would refer to a difference in response to a given amount of potassium at different magnesium levels, and not to effects of variation in the relative concentrations. Clearly when the sum of the two nutrients is constant no information of this kind is available, and it is impossible to determine from the figures alone whether the observed differences are due to either of the variables alone, to simple summations of unknown effects of both, or finally to interaction between the two factors. Herein lies the great weakness of such designs. From a factorial experiment, on the contrary, main effects of the ions and all their interactions may be directly stated and assessed. Neither can the methods of correlation be satisfactorily used with triangular arrangements. Thus in the cation triangle, Lyon *et al.* (53) correlated the percentage of diseased fruit with calcium supply, obtaining a highly significant relationship. Since, however, calcium supply is by the design completely correlated with the sum of the other two cations, disease is just as closely related to this sum as to calcium. While there may be other reasons for the belief that the primary relationship is with calcium, without assumptions that may or may not be justified there is no means of determining this from the data.

In this review criticism has been levelled against the designs of some experiments and the mode of interpretation of the results obtained. In view of the importance of the issues this attitude is necessary, since however fundamental the aim of the investigation, and however carefully the experiment is conducted, the value of the work depends finally on the amount and reliability of the information contributed, and this is largely determined by the adequacy of the experimental design, together with statistical confirmation of the results.

LITERATURE CITED

1. Broyer, T. C., and Hoagland, D. R., *Am. J. Botany,* **30,** 261–73 (1943)
2. Routien, J. B., and Dawson, R. F., *Am. J. Botany,* **30,** 440–51 (1943)
3. Converse, C. D., Gammon, N., and Sayre, J. D., *Plant Physiol.,* **18,** 114–21 (1943)
4. Coleman, R., *Soil Sci.,* **54,** 237–46 (1942)
5. Graham, E. R., and Albrecht, W. A., *Am. J. Botany,* **30,** 195–98 (1943)
6. Commoner, B., Fogel, S., and Muller, W. H., *Am. J. Botany,* **30,** 23–28 (1943)
7. Commoner, B., and Mazia, D., *Plant Physiol.,* **17,** 682–85 (1942)
8. Went, F. W., *Plant Physiol.,* **18,** 51–65 (1943)
9. Wallace, T., *The diagnosis of mineral deficiencies in plants,* 116 pp. (H. M. Stationery Office, London, 1943)
10. Ulrich, A., *Soil Sci.,* **55,** 101–12 (1943)
11. Emmert, E. M., *Kentucky Agr. Expt. Sta. Bull.,* **430,** 48 pp. (1942)
12. Scarseth, G. D., *Soil Sci.,* **55,** 113–20 (1943)
13. Cullinan, F. P., and Batjer, L. P., *Soil Sci.,* **55,** 49–60 (1943)
14. Nightingale, G. T., *Botan. Gaz.,* **103,** 409–56 (1942)
15. Nightingale, G. T., *Botan. Gaz.,* **104,** 191–223 (1942)
16. Arnon, D. I., *Ann. Rev. Biochem.,* **12,** 493–528 (1943)
17. Nightingale, G. T., *Soil Sci.,* **55,** 73–78 (1943)
18. Arnon, D. I., and Hoagland, D. R., *Botan. Gaz.,* **104,** 576–90 (1943)
19. Olsen, S. R., and Shaw, B. T., *J. Am. Soc. Agron.,* **35,** 1–9 (1943)
20. Willcox, O. W., *J. Am. Soc. Agron.,* **35,** 454–59 (1943)
21. Various Authors, *Soil Sci.,* **55,** 1–126 (1943)
22. Albrecht, W. A., *Soil Sci.,* **55,** 13–21 (1943)
23. Chu, S. P., *J. Ecol.,* **30,** 284–325 (1942)
24. Peech, M., and Bradfield, R., *Soil Sci.,* **55,** 37–48 (1943)
25. Pierre, W. H., and Bower, C. A., *Soil Sci.,* **55,** 23–36 (1943)
26. Schroeder, R. A., and Albrecht, W. A., *Soil Sci.,* **53,** 481–88 (1942)
27. Hunter, A. S., *Soil Sci.,* **55,** 361–69 (1943)
28. Hunter, A. S., Toth, S. J., and Bear, F. E., *Soil Sci.,* **55,** 61–72 (1943)
29. Richards, F. J., *Ann. Botany, n.s.,* **5,** 263–96 (1941)
30. Chapman, H. D., and Brown, S. M., *Soil Sci.,* **55,** 87–100 (1943)
31. Richards, F. J., and Shih, S., *Ann. Botany, n.s.,* **4,** 403–25 (1940)
32. Mullison, W. R., and Mullison, E., *Plant Physiol.,* **17,** 632–44 (1942)
33. Mullison, W. R., *Plant Physiol.,* **16,** 813–20 (1941)
34. Lehr, J. J., *Soil Sci.,* **53,** 399–411 (1942)
35. Carlson, W. E., *Soil Sci.,* **54,** 425–38 (1942)
36. Watson, D. J., and Russell, E. J., *Empire J. Exptl. Agr.,* **11,** 49–77 (1943)
37. Harmer, P. M., and Benne, E. J., *J. Am. Soc. Agron.,* **33,** 952–79 (1941)
38. Lineberry, R. A., and Burkhart, L., *Plant Physiol.,* **18,** 324–33 (1943)
39. Mandels, G. R., *Plant Physiol.,* **18,** 449–62 (1943)
40. Thomas, M. D., Hendricks, R. H., Collier, T. R., and Hill, G. R., *Plant Physiol.,* **18,** 345–71 (1943)
41. Eaton, S. V., *Plant Physiol.,* **17,** 422–34 (1942)

42. Eaton, S. V., *Botan. Gaz.*, **104**, 82–89 (1942)
43. Eaton, S. V., *Botan. Gaz.*, **104**, 306–15 (1942)
44. Wood, J. G., and Petrie, A. H. K., *Australian J. Exptl. Biol. Med. Sci.*, **20**, 249–56 (1942)
45. Wood, J. G., *Australian J. Exptl. Biol. Med. Sci.*, **20**, 257–62 (1942)
46. Welker, E. L., and Wynd, F. L., *Plant Physiol.*, **18**, 498–507 (1943)
47. Mason, T. G., and Phillis, E., *Ann. Botany, n.s.*, **6**, 455–68 (1942)
48. Phillis, E., and Mason, T. G., *Ann. Botany, n.s.*, **7**, 147–56 (1943)
49. Mason, T. G., and Phillis, E., *Ann. Botany, n.s.*, **7**, 157–69 (1943)
50. Richards, F. J., *Ann. Botany, n.s.*, **8** (In press)
51. Hamner, C. L., *Botan. Gaz.*, **101**, 637–49 (1940)
52. Hamner, K. C., Lyon, C. B., and Hamner, C. L., *Botan. Gaz.*, **103**, 586–616 (1942)
53. Lyon, C. B., Beeson, K. C., and Barrentine, M., *Botan. Gaz.* **103**, 651–67 (1942)

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GROWTH-REGULATING SUBSTANCES IN PLANTS

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Auxins have been made the main topic of this discussion in which the reviewer has attempted to be informative rather than comprehensive. Papers have been selected from those which have appeared since the last review on auxins in this publication in 1939 (1). The subject has been divided into four parts: (*a*) terminology; (*b*) the different forms of auxin and their analysis; (*c*) the action of auxin; and (*d*) practical aspects.

Terminology

Phytohormone.—Our increased knowledge in the field of growth-regulating substances has made it necessary to redefine our terms. Some terms such as "plant hormone" already have acquired through usage a meaning different from that which was originally intended. Such changes, which naturally occur, have to be sanctioned by general agreement in order to avoid confusion.

The term phytohormone or plant hormone is used in at least two different senses: (*a*) in its restricted sense as a naturally occurring correlation carrier, "a hormone is a substance which, being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process" (2); and (*b*) in a broad sense (3, 4), ". . . . growth-regulating substances generally called plant hormones . . . ." (3). According to the first interpretation the convenient term phytohormone cannot be applied to many synthetic substances which have not been shown to be plant products, yet have a physiological activity similar to that of the natural substance. Thus, naphthaleneacetic acid would not be a hormone, whereas indoleacetic acid might be classified as such. In addition it is very difficult to decide whether or not a substance is naturally occurring. For instance, indoleacetic acid which was long considered to be absent in higher plants has now been conclusively demonstrated to be present (5, 6).

Clearly the term phytohormone, in its restricted sense, has become impractical. However, since the term is descriptive (literally meaning activator) and brief, it should not be dropped from the literature as

Schopfer (7) suggests, but the reviewer would be in favor of redefining the term and making it synonymous with growth-regulating substances, as is already being widely done. In this form a phytohormone might be defined as an organic substance, other than the traditionally recognized energy-supplying substances, which regulates physiological functions in plants. This definition includes synthetic as well as natural substances, "vitamins" as well as "hormones," growth inhibitors as well as growth promoters. It includes also "local activators" such as wound hormones as well as typical "correlation carriers"—substances which are produced in one part and act in another—such as auxins.

Often it is assumed that phytohormones act in minute amounts and concentrations. Although this is often the case, there are exceptions. For instance traumatic acid, a wound hormone of plants, has a detectable activity at a concentration of 10 p.p.m. and a maximal response at concentrations ten to twenty times higher (8). On the other hand, it is known that sugar cane will take up a maximal amount of phosphate when the phosphate concentration in the nutrient is as low as 1 p.p.m. P_2O_5 per l. (9). Hence for maximal response the macronutrient phosphate is required in 100 times lower concentrations than the hormone traumatic acid. For similar reasons Williams (10) has dropped the term "small amounts" from the definition of vitamin. Ascorbic acid, one of the earliest recognized vitamins, appears to be necessary in the diet in amounts of the same order of magnitude as calcium.

Hormone, vitamin, nutrilite.—In the earlier days of research on vitamins and hormones these two groups of substances were seemingly very different. Vitamins were classified as accessory nutrients, hence exogenous substances. Hormones by contrast were endogenous substances. Recently, however, it has become clear that some vitamins function as coenzymes[1] and at the same time can be considered hormones. Moreover, one and the same substance may be a hormone for one organism and a vitamin for another (11, 12). Clearly then, the actual source of a substance is of no value for classification (7). However, Schopfer (7) still attempts to distinguish between growth factors of vitamin nature and those of hormonal nature, the distinction being that the vitamins act on the intermediate metabolism

[1] For a general discussion see (7). For thiamin involved in reactions other than decarboxylation of pyruvates see (31), and for a possible role of biotin as a coenzyme see (30).

(e.g., thiamin), while the hormones, in the final analysis, act specifically on the morphology of the organism (e.g., auxin). But this classification also may not long survive, because according to Commoner & Thimann (13) auxins are involved in respiration, perhaps as coenzymes (11, 14, 15). Hence auxins which are cited as examples of hormones by Schopfer would be involved in intermediate metabolism. In addition the reviewer is not convinced that a fundamental difference exists between a root growing *in vitro* under influence of thiamin and an *Avena* coleoptile elongating under the influence of auxin. In the final analysis both factors are acting on the morphology of the organs involved.

Williams (10) defines vitamins as "nutritionally essential organic substances other than the traditionally well recognized proteins, lipides and carbohydrates and their constituent parts." This definition is applicable to the field of animal nutrition for which the term vitamin was originally proposed. Earlier Williams (16) proposed the term nutrilites, the use of which was intended to include plants as well, in the following words: "We may then define a nutrilite as a substance other than the well recognized nutrients, which functions in small amounts in the nutrition of organisms." Later (10) Williams dropped the term "small amounts," but Schopfer (7) adheres to it in his definition of vitamin.

An interesting observation was made by Doudoroff (17) which has a bearing upon the determination of nutritionally essential substances. In his studies on the nutrition of *Pasteurella pestis* it was found that when leucine was present in the medium, the addition of isoleucine or valine was necessary. Yet neither of these substances appeared necessary when leucine was omitted from the medium. Thus the "elimination method" which is usually employed for finding minimum nutritional requirements has its dangers, because there seem to be cases in which the elimination of a constituent prevents growth, not because it is indispensable, but because it counteracts the toxic effect of another substance in the mixture. A similar case is known in mineral nutrition also. Liebig, Vanselow & Chapman (18) found that in citrus plants aluminum was necessary only when toxic amounts of copper were present in the nutrient solution. With copper present in amounts below the toxic level aluminum was without effect.

Auxin.—This term is a generic name for those substances which bring about growth by elongation as measured by the *Avena* test. Hence naturally-occurring as well as synthetic substances may be

auxins as long as they show activity in this test. As such this term is much more useful than the term phytohormone in the restricted sense. Perhaps the term auxin is the most precisely defined term in the field. However, this does not necessarily make it entirely satisfactory. It so happened that the definition was made before more suitable tests for elongation (19, 20, 21, 22) were developed and hence the term auxin is not entirely synonymous with elongation-controlling substance, as might be desirable.

Since auxins have been shown to have physiological activity other than the control of elongation, such as for instance the production of parthenocarpic fruit, there is danger that the term auxin will be carried over to substances which produce parthenocarpic fruit but which do not react in the *Avena* test. Such a substance is naphthoxyacetic acid, (23 to 26) which, together with a score of other substances, was tested by Gustafson (27) in seven different tests. Naphthoxyacetic acid gave the best activity of all substances tested for parthenocarpy, but it failed to react in the *Avena* test. Neither did it react in the deseeded *Avena* test (28). Hence the substance is not an auxin, but it is a growth-regulating substance and a "phytohormone" if one accepts the term in its broad sense as outlined above.

Terms such as growth-regulating substance and growth factor are general terms and self explanatory. The expressions growth substance and growth hormone are often used as synonyms for auxin. In analogy with the term isomer, the term isotel (from the Greek, "same purpose") has been proposed for chemically distinct substances which perform the same function (29). Vitamer is a similar term but restricted to substances replacing vitamins (30).

Forms of Auxin in Plants and Their Analysis

Auxins occur in plants in a variety of forms and combinations. During a discussion at the *Symposium on Hormones at Cold Spring Harbor* in 1942 Skoog (32) summarized the present status of this problem. He envisaged auxins occurring in the plant

> . . . . as existing in three forms: 1) bound inactive auxin, 2) an active auxin complex and 3) free auxin in equilibrium with the first two forms. The bound auxin is evidently of two types: 1) the so-called precursor existing especially in seeds and other storage organs. This auxin is liberated in free form by hydrolysis at alkaline pH, by heat, by the action of ether, etc. 2) A complex present in leaves and probably in green tissues generally. . . . . Its behavior indicates it to be a protein complex.

Closely connected with these different types of auxin are the various techniques by which auxin is extracted from plant materials. The chemist extracting material is primarily interested in a good yield, the physiologist on the other hand wants to know what the extracted substance represents in the living plant. For this reason various techniques have been devised, which the reviewer has ventured to group together in a single scheme (Table I), in the hope that it might show how they are related to one another. The existence of endosperm precursor, of a proteinaceous auxin complex in leaves, and of free auxin has been conclusively demonstrated. There is reason to expect a fourth form of auxin, an active auxin complex referred to as "bound auxin" by Went & Thimann (2), although the properties ascribed to it (2, 33) are highly speculative.

TABLE I

FORMS OF AUXIN AND THEIR ANALYSIS

ENDOSPERM PRECURSOR (inactive, nonprotein; wheat, corn, coconut)	$\longrightarrow$ / $\longleftarrow$?	FREE AUXIN	$\longrightarrow$ / $\longleftarrow$?	ACTIVE AUXIN COMPLEX ("bound auxin")
LEAF PRECURSOR (inactive, protein; spinach, *Lemna*, tobacco, *Ulva*, green material in general, coleoptiles?)	$\longleftarrow$? / $\longrightarrow$			

Methods for complete auxin extraction of material containing endosperm precursor:	Methods for complete auxin extraction of material containing leaf precursor:	Methods for the exclusive extraction of free auxin:
(*a*) hydrolysis by alkali, heat, etc. (5, 6, 34, 35)	(*a*) proteolytic enzymes (39, 48, 49)	(*a*) ether extraction of heat-treated material (46, 48)
(*b*) human digestion (6)	(*b*) wet ether extraction of long duration (39, 46, 48)	(*b*) short period ether extraction of coleoptiles (48, 61)
(*c*) ether treatment of coconut milk (44).	(*c*) exhaustive diffusion of coleoptile tips (61).	(*c*) short period cold water extraction (35).

How much justification there is for distinguishing sharply between endosperm precursor and leaf precursor, is a matter which will be decided by future research. The reason for this distinction lies mainly in the different ways in which they yield active auxin. The endosperm precursor according to Avery *et al.* (34 to 38) and Haagen-Smit *et al.*

(5, 6) yields auxin readily upon hydrolysis with alkali, whereas according to Thimann, Skoog & Byer (39), the leaf precursor yields auxin upon digestion with proteolytic enzymes and not when treated with alkali. However, recently Gordon & Wildman (40) have shown that considerably greater yields of auxin can be obtained from cytoplasmic leaf proteins of spinach by hydrolysis with alkali than by enzymatic digestion. Moreover, Haagen-Smit *et al.* (6) reported some auxin release by enzymatic digestion from endosperm material. It may very well be, therefore, that the distinction between the two classes of precursors is not as definite as one might conclude from the diagram.

Endosperm precursors.—It has been known for some time (41, 42, 43) that endosperm of maize and other cereals yields considerable amounts of auxin when extracted with water, but little or none when extracted with organic solvents. Extensive investigations along this line have been carried out by Avery *et al.* (34 to 38), and by Haagen-Smit *et al.* (5, 6) and the following general conclusions can be drawn from the work of these two groups. (*a*) The auxin precursor of the endosperm can be readily hydrolyzed into free (i.e., active) auxin. This can be most effectively accomplished by keeping the extract at pH 10. Avery, Berger & Shalucha demonstrated that at this pH hydrolysis goes to completion when the extract is boiled for only one minute (35). (*b*) The precursor is not soluble in ether or chloroform but is readily soluble in water, ethyl alcohol, and methyl alcohol. It cannot be a very large molecule since it passes readily through a cellophane dialyzing membrane (35). (*c*) The end product of hydrolysis by means of alkali is largely indoleacetic acid which was obtained in crystalline form (5, 6). No active auxin of the type of auxin-*a* was found, which was to be expected since this auxin is not stable at alkaline pH. However, the presence of pseudoauxin-*a* was reported in corn meal (6), while an alkali-labile auxin was found in wheat (37).

Very high auxin yields were obtained from wheat flour which was eaten and the auxin recovered in the urine (5, 6). The auxin yield obtained by this method was 6.9 mg. indoleacetic acid equivalents per kg. of wheat. This compared favorably with the yield obtained by hydrolysis *in vitro* at pH 10.5 which yielded 6.96 mg. indoleacetic acid equivalents per kg. of wheat.

In coconut milk an auxin precursor has been found (44) which has many properties of the precursors just described. It is water soluble, is hydrolyzed at alkaline pH and by autoclaving, and in addi-

tion passes through a cellophane dialyzing membrane and must, therefore, have a fairly small molecular size. The coconut precursor reacts with ether by quickly yielding large amounts of free auxin. An agar block soaked in fresh coconut milk gave a curvature of only 0.6° in the *Avena* test. After the milk was shaken with ether, and the ether fraction taken up in a volume of water equal to the original volume, agar blocks soaked in this solution gave a curvature of 21.5°—a thirty-five-fold increase.

Nonendosperm precursors.—Soon after auxin extraction methods were used for plant physiological investigations, it was found that plant material continued to give off auxin during extraction with solvents such as ether (39, 45 to 48). *Helianthus* seeds, tomato seedlings, *Lemna* fronds, *Ulva* fronds, etc., continued to yield auxin for many months after the extraction was started. Neither grinding nor hydrolysis with acid or alkali (39, 48) could speed up the procedure. Skoog & Thimann (47) showed that treatment with chymotrypsin greatly increased the auxin yield from *Lemna* fronds as well as tobacco leaves (39) and suggested that the auxin is released from a protein complex. Wildman & Gordon (49) actually isolated leaf proteins from spinach which yielded auxin upon digestion with proteolytic enzymes, while no auxin was extracted when these proteins were treated with ether. However, in a later paper (40) they found that treatment with alkali will yield auxin from these proteins, as well as from tryptophane. Thus, it was conclusively demonstrated that auxin-protein complexes occur which contain auxin.

Thimann & Skoog (39, 48) carried out extensive investigations on the extraction of auxin from *Lemna* fronds. They found that it takes approximately sixty days of continuous ether extraction to extract the auxin completely. Boiling stopped the process and dried material failed to yield any auxin, which finding is in agreement with results obtained by Link *et al.* (50) and by Gustafson (46). The reason that auxin cannot be extracted from dry material may be both physical and chemical (48). Dried material will again yield auxin upon wetting. When dried over Na_2SO_4 in the cold no reduction in auxin yield is observed when the dried material is wetted before extraction, but when dried in hot air the final yield is considerably reduced. Both Went and Cooper (unpublished) have observed that the auxin yield of mature leaves upon extraction with ether is considerably increased when the leaves are dried. Went (personal communication) believes this to be due to reduced auxin destruction.

Thimann, Skoog & Byer (39), on the other hand, attribute this to enzymatic liberation of auxin during the process of slow drying. They found that when tomato leaves are frozen and dried quickly, the auxin yield is not increased by drying. Depending upon the conditions of drying, both auxin destruction and enzymatic liberation might be involved.

Tryptophane is a precursor which may be discussed under this heading. Skoog (51) demonstrated that it showed no activity in the "standard" *Avena* test but that it responded in the "deseeded test," which is attributed to a slow oxidative deamination into indoleacetic acid in the test plant. The growth reactions which Kraus (52) reported with tryptophane in bean plants are also very likely due to the same slow release of indoleacetic acid. The rapid activity on *Avena* coleoptiles of tryptophane-lanolin mixtures reported by Stewart (53) may be attributable to partial transformation into auxins during the preparation of the mixtures which involved prolonged heating. Gordon & Wildman (40) demonstrated the instability of tryptophane under such conditions.

Mitchell & Stewart (54) compared growth responses caused by naphthaleneacetic acid and its amide in the pea test, on *Avena* coleoptiles and on other objects and found that in general the two substances cause the same response, but that the amide is slower. Also this may be attributable to a slow conversion of the amide into the acid.

Another precursor, isolated from leafy material, is a preparation of a growth inhibitor (55, 56) which was found to liberate auxin upon hydrolysis or when placed in contact with cut plant surfaces.

Active auxin complex.—Since it is reasonable to assume that free auxin has to associate with some cell component in order to cause elongation, the concept of "bound auxin," in a sense different from precursor, has been in the minds of many investigators.[2] It has been discussed by Went & Thimann (2) and recently by Thimann (15), Skoog *et al.* (14), and by Went who elaborated on the properties it might have (33). When Commoner & Thimann (13) found that auxin increased the rate of respiration of plant tissue in the presence

[2] Although the theory that auxin acts by combining chemically with other cell components is widely adhered to, the possibility remains that under certain conditions the action of auxin might be of a physicochemical nature, such as affecting interfacial tensions in the living system. Such action was proposed in one of the many attempts to explain the role of auxin in bud inhibition (57). Ascorbic acid is often thought of as a physicochemical agent rather than as a coenzyme (7, 58).

of malate, the concept received new impetus. It was thought that perhaps auxin might be a coenzyme for the four-carbon acid respiratory cycle (15). This would make "bound auxin" a protein-auxin complex.

The protein-auxin of Wildman & Gordon (49) does not yield auxin upon ether treatment, whereas *Lemna* fronds, which according to Skoog & Thimann (47) contain an auxin-protein complex, yield auxin only slowly when extracted with ether. The "bound auxin" on the other hand is alleged (2, 33) to give off its auxin readily to organic solvents. The only type of bound auxin, so far known, which yields free auxin readily when extracted with ether is the one occurring in coconut milk (44). This form, however, passes through a dialyzing membrane and therefore must have a smaller molecular size than the typical proteins.

Before trying to match the properties of "bound auxin" with those of the better known forms of auxin, one would do well to know if the properties assigned to it rest on a fairly secure basis, as far as this is possible for a hypothetical compound. When one investigates the property assigned to "bound auxin" (2, 33) of yielding auxin upon treatment with ether, one finds that the evidence is negative. Thimann (59) found that the basal regions of *Avena* seedlings yielded auxin by chloroform extraction, but not by diffusion into agar. This was construed as "evidence that the auxin in the plant is present in at least two different forms: the free moving auxin which can be collected by the diffusion method, and the bound auxin, which is obtained, together with the free auxin, by extraction of the tissues" (2, p. 68). In the light of later investigations, however, this evidence is much weaker than it originally appeared. (*a*) Later workers using more sensitive techniques did obtain auxin by diffusion from coleoptile sections (51, 60). (*b*) Auxin destruction at cut surfaces of sections cut from basal regions of seedlings is considerably higher than in those cut from apical regions (42, 43). (*c*) According to a recent finding, the possibility exists that no measurable amount of auxin is given off by diffusion unless new molecules are present (by production or application) to "push" the existing ones out of the section (61).

The extraction of free auxin.—The extraction method was originally employed by physiologists to give information regarding the actual auxin concentration in the plant, whereas the diffusion method was thought to yield data on the production of auxin. However, it seems that only in coleoptiles can auxin be extracted completely in a short period of time (48, 61). This auxin probably represents the

active auxin in the plant at the time of extraction. Some caution should be observed in accepting this conclusion since conversion may occur during extraction and since in coconut milk an auxin precursor is known which yields active auxin readily upon extraction with ether.

In plant materials other than coleoptiles, determinations of active auxin may be possible only in heated material. Here the extraction yields what was initially free auxin, together with any auxin set free during the heating of the tissue (48). A method, which appears to be generally applicable for the determination of free auxin in plant materials, is the one devised by Gustafson (46). According to this method the tissue is finely ground in dry ice to prevent destruction of the auxin. Next, the powdered tissue is immediately boiled for one minute to prevent activation of the precursor. This was later confirmed (39). The water as well as the residual plant material is shaken with ether to remove the active auxin. In *Iris* ovules, all the auxin was found in the water fraction.

The relation of free auxin to precursor.—In all tissue so far investigated, including storage tissues, an amount of free auxin is found which is many times smaller than the amount of auxin which can be activated from precursor present in these tissues. Most data indicate a precursor content of about 90 to 95 per cent of the total amount of auxin potentially available and hence an active auxin content of 5 to 10 per cent. Since, in determinations of free auxin, there is always the danger of activation of the precursor, 5 to 10 per cent active auxin in tissues is likely to be a maximal figure. It will be clear then that most tissues have a considerable store of reserve auxin, the physiological significance of which for tumor and fruit development will be discussed in a later section.

The methods by which the above figures were computed were different for various tissues. The extreme coleoptile tip of maize was used for a study of the total amount of auxin that could be obtained by diffusion from the living tip as compared with the total amount that could be extracted with ether (61). Such tips continued to give off auxin for a period of twelve hours. The total amount of auxin collected during this period was some twelve times larger than the total amount obtained from fresh tips by ether extraction. Tips which had stopped giving off auxin by diffusion were still found to yield approximately as much auxin by extraction as fresh tips. This residual auxin might be "bound auxin" in the sense of Went & Thimann. However, as an alternative explanation it was suggested that auxin is

only given off by diffusion into agar when new active auxin molecules are released from precursor. These newly formed molecules push the existing ones along their path of transport and out of the tip into the agar block. When the supply of precursor in the tip is exhausted and no more new auxin molecules are formed, the last ones formed are consequently trapped between their place of origin and the cut surface. The auxin collected by complete diffusion plus that left behind and recovered by extraction is the total potential auxin content of the coleoptile tip. Of this amount only about 8 per cent is present as active auxin in a freshly cut tip. The concentration of this active auxin was found to be 335 μg. indoleacetic acid equivalents per kg. fresh weight of tips, which is a high figure since a concentration of 100 μg. per l. gives a maximum angle in the *Avena* test. Somewhat similar figures for auxin content were obtained for *Avena* coleoptiles (61).

In the previously mentioned investigations of Thimann & Skoog (48), *Lemna* leaves were exhaustively extracted with ether, a procedure which took over sixty days to reach completion. In other series, leaves were boiled and then extracted with ether. The first procedure should yield data for total potential auxin content; the second procedure might yield the approximate amount of active auxin present at the time of extraction. A calculation from their data by the reviewer shows an active auxin content of about 3 per cent and precursor content of 97 per cent of the total potential amount of auxin in *Lemna* fronds.

From a comparison of water extraction of maize endosperm of short duration at low temperature and low pH with complete extraction by hydrolysis with alkali, Avery, Berger & Shalucha (35) drew the conclusion that 10 per cent is present as free auxin and 90 per cent as precursor. They also (38) found that this ratio held for six different types of maize endosperm (brittle, waxy, floury, sugary, popcorn, and Canada flint). The precursor content varied only between 87 and 92 per cent of the total potential auxin, although the absolute amounts of total auxin varied between 132 (sugary) and 32 (popcorn) μg. of indoleacetic acid per gm. dry weight.

Hydrolyzed and ether-treated coconut milk (44) could be made to yield approximately fifty times more auxin than was found to be present in the fresh state. Hence here too we find 2 per cent free auxin against 98 per cent precursor.

The chemical nature of free auxin.—For many years it was thought that the native auxin of higher plants was auxin-*a* or -*b* or their de-

rivatives, while the lower plants produced indoleacetic acid. Recent investigations necessitate a revision of this thesis. The most conclusive demonstration of indoleacetic acid in higher plants was the isolation of the crystalline compound from corn meal by Haagen-Smit, Leech & Bergren (5). In addition a variety of investigations on other plant material pointed to the presence of indoleacetic acid in higher plants. Diffusion coefficient determinations and acid-alkali sensitivity tests showed that the auxin of tomato leaves (62) and of spinach proteins (49), the auxin released from the inhibitor of radish cotyledons (work by Redemann, reported in 5), and the auxin of the giant kelp *Macrocystis* (63) all resembled indoleacetic acid rather than auxin-*a*. The only exception (in recent years) was found in the auxin of isolated pea roots growing *in vitro,* which showed the characteristics of auxin-*a* (64). In the work on diffusion coefficients of auxins it was reported that the values came out somewhat higher (consequently giving a lower molecular weight) than could be expected for pure indoleacetic acid (49, 63). The reason for this might be a slight depressing effect of relatively slow-diffusing inhibitors present in the impure preparations used. This would affect the apparent distribution of the auxin in the diffusion test.

Units.—Since indoleacetic acid has now been established as a widely occurring auxin in plant tissues and since this acid is easily obtainable in the pure form, it seems that auxin activity of extracts should be expressed in terms of indoleacetic acid (38, 46, 63, 65). The continued use of other arbitrary units no longer seems justifiable in the light of our present knowledge.

Action of Auxin

Effect of auxin on respiration, elongation, and cyclosis.—During recent years evidence has accumulated which shows that auxin is involved in the process of respiration. Commoner & Thimann (13, 15) found that when coleoptile sections, starved by soaking in water, were treated with either malic or fumaric acid, the addition of auxin produced a marked increase in the respiratory rate. Since the presence of the 4-carbon acids was necessary to evoke this reaction, it was concluded that auxin catalyzes a respiratory system involving these acids. The increased respiration was found to parallel the increase in elongation of coleoptile sections. In addition, low concentrations of the dehydrogenase inhibitor iodoacetate was found to inhibit growth completely, while it lowered respiration only by 10 per cent. Hence it is

possible that the respiratory system involving auxin accounts for only a small part of the total respiration, yet it may control a substantial part of the growth process.

Further evidence of stimulation of respiration by auxin was given in a preliminary report by Berger & Avery (65a), who showed that the activity of alcohol dehydrogenase and malic dehydrogenase of coleoptile sections was increased respectively 200 and 150 per cent when the sections were treated with relatively high concentrations (10 mg. per l.) of indoleacetic acid. Previous attempts to find such stimulations in cell free extracts had met with failure (65b). In view of their findings that alcohol dehydrogenase is more sensitive to iodoacetate than malic dehydrogenase, they suggest that alcohol dehydrogenase activity is closely concerned with control of growth and that auxin controls growth by virtue of activating this enzyme.

It had earlier been found by Sweeney & Thimann that a close parallel exists between the acceleration of the protoplasmic streaming in the *Avena* coleoptile on the one hand and respiration and growth on the other. They (66, 67) developed an elegant apparatus for continuous recording of the rate of streaming and demonstrated that auxin will only produce an acceleration of the rate of streaming if malate is present. The basal rate of streaming is not affected by auxin. Iodoacetate was found to affect the acceleration of streaming in a fashion similar to its effect on elongation and respiration, which is further evidence for a parallelism between the effects of auxin on streaming, elongation, and respiration, all of which are apparently mediated by the 4-carbon acid system. The streaming in root hairs of *Avena* is also stimulated by indoleacetic acid, but at concentrations 10,000 times lower (10^{-4} to 10^{-5} mg. per l.) than for coleoptiles (68). Northern (69) concluded from measurements of the displacement of chloroplasts and starch grains after centrifuging that applications of auxins reduce the protoplasmic viscosity in bean stems and petioles. This may be correlated with the acceleration of protoplasmic streaming.

Auxin and water uptake.—Since water uptake is the driving force of elongation, Commoner *et al.* (70) attempted to investigate further the mechanism of elongation by studying the water uptake of potato tissues. Such tissues show an increased water uptake under the influence of auxin, which was first clearly demonstrated by Reinders (71). This increased water uptake involves oxygen and since the auxin-treated tissues at the end of the experiment had a lower dry

weight, which was not due to exosmosis of sugar, it was concluded that the auxin promotes the respiration as well as the water intake (71). It has been shown that a variety of tissues and organs show an increased water content when treated with auxin (72, 73, 74).

Commoner *et al.* investigated potato tissues in 0.2 *M* sucrose solution and found that after 160 hours the sections had lost 30 per cent in wet weight. In the presence of indoleacetic acid, however, no water loss occurred, while if in addition to indoleacetic acid, potassium chloride or fumarate was added a 10 per cent weight increase was recorded. This was interpreted to indicate that ". . . . the auxin-four-carbon acid system regulates the active absorption of water, and so causes cell elongation. This effect is probably due to the influence of the auxin system on the absorption of salt by plant cells." This conclusion seems rather improbable since Reinders found the increased water uptake under influence of auxin to take place in distilled water. However, it is still possible that an increased osmotic pressure brought about by an increased hydrolysis of starch would cause the increased water uptake. Many instances are known where auxin has been shown to promote the hydrolysis of starch (75 to 78) and suggestions toward its possible role in growth processes have been made (79).

In view of the fundamental importance of the problem, experiments were undertaken (80) in order to investigate whether or not the increased water uptake of potato tissues is brought about by an increased osmotic pressure. The results showed that the expressed sap of auxin-treated tissues, which still were in the process of water uptake, had an osmotic pressure (determined cryoscopically) which was significantly lower, and not higher, than that of the untreated controls. This clearly indicates that the increased water uptake is not caused by an increased osmotic pressure of the cell sap.

Two other alternatives are possible[3] to account for the increased water uptake of potato tissues under influence of auxin: (*a*) A decreased wall pressure. This is a possibility because of the thin walls of the tissue involved. (*b*) An increase in nonosmotic water uptake. Such nonosmotic water uptake has been reported by Lyon (82) for potato tissue. It also occurs in tomato roots (83), and is also thought

[3] The pressure with which plant cells take up water may be expressed by the equation: $S.P. = O.P. + A.P. - W.P.$ In which *S.P.* is the suction pressure of the cells, also referred to as *D.P.D.* or diffusion pressure deficit (81); *O.P.* is the osmotic pressure; *A.P.* is the "active" pressure (82, 83, 84) which has been added to the usual equation (81); *W.P.* is the wall pressure.

to be involved (Anderson, private communication) in the large water-absorbing power of young cotton bolls (84). The possibility exists that this nonosmotic water uptake is controlled by auxin. Skoog, Broyer & Grossenbacher (85) reported a stimulating effect of auxin on the rate of exudation of decapitated *Helianthus* grown in nutrient solution. Since on the one hand, auxin increases the amplitude of the diurnal periodicity of exudation (85) and, on the other hand, it has been demonstrated (83) that the nonosmotic component of the root pressure (and not the osmotic one) shows the diurnal periodicity, it is possible that auxin is involved in the nonosmotic water uptake of plants. However, direct evidence is lacking at present. If such an effect of auxin could be demonstrated, the possibility exists that it would operate coordinately with the lowering of the wall pressure (*W.P.*) in bringing about elongation of cells.

Interaction between auxin and chemically related substances.—While analyzing the mechanism of the pea test, Went (20) discovered that its sensitivity to auxins was considerably increased by the action of substances chemically related to auxins but not active as such. This led to a considerable controversy as to the mechanism of this action and will be briefly reviewed because of its possible importance for the understanding of the growth process as a whole.

When split pea stems are soaked in phenylbutyric acid or cyclohexaneacetic acid their growth is not increased over that of the controls. However, if these stems are subsequently treated with low concentrations of indoleacetic acid, those pretreated with phenylbutyric acid or some other compounds showed a much greater auxin response than the nonpretreated controls. Went demonstrated that this process involves two separate reactions: (*a*) a conditioning of the split stem such as produced by phenylbutyric acid, called "preparatory reaction," and (*b*) the elongation reaction itself. Phenylbutyric acid can only take part in reaction (*a*) and is inactive in reaction (*b*). Indoleacetic acid on the other hand is active in both reactions. Went for this reason called substances which will only react in reaction (*a*) "hemi-auxins." Although indoleacetic acid can take part in both reactions, a higher concentration is required for reaction (*a*) than for reaction (*b*). In addition, reaction (*a*) was shown to be pH insensitive while reaction (*b*) is pH sensitive. Went therefore clearly demonstrated that in the elongation of split pea stems, pretreated with "hemi-auxins," two separate reactions are involved. He concluded that the two reactions are chain reactions and that both reactions are part of the normal process

of elongation. In another paper (86) this idea was further expanded. "Hemi-auxins" were applied to the tip of intact *Avena* coleoptiles and after this pretreatment the plant was cut into sections which were left to elongate in an indoleacetic acid solution. It was found that the sections cut from the apical part of pretreated coleoptiles showed an increased elongation over those cut from the controls, whereas the sections cut from the basal regions usually showed an inhibition relative to the controls. From this Went concluded that in reaction (*a*) the "hemi-auxins" (and by inference auxins as well) redistributed other growth factors, called elsewhere "calines" (87), and that in reaction (*b*) the auxins react with these other growth factors to give cell elongation. The inhibition of the sections cut from the lower regions of the coleoptile is explained as being due to a temporary shortage of the food factors which were moved to the apical regions under influence of the "hemi-auxins" employed in the pretreatment.

Although Went demonstrated the presence of two separate reactions, it does not follow that these reactions are part of a chain reaction, since they might be two parallel reactions. In addition, the sensitizing reaction need not be a "preparatory reaction" of the regular process of elongation, but simply an auxin-sparing reaction caused by inactivation of destructive enzymes by the "hemi-auxins." In other words interaction between auxins and other inactive but chemically related substances may be a question of competitive action. It is along such lines that Skoog *et al.* (14) criticized Went's conclusions. They showed that when a mixture of phenylbutyric acid and indoleacetic acid is given to *Avena* coleoptiles, the action of the auxin is considerably inhibited by the presence of phenylbutyric acid. In experiments with coleoptile sections they found the same effect except that with relatively low concentrations of auxin and relatively high concentrations of phenylbutyric acid a growth-promoting effect was found comparable (but smaller in magnitude) to the sensitizing effect described by Went (20) in the split pea stems. Skoog *et al.*, however, explained the effect as being due to an auxin-sparing action of phenylbutyric acid. Since it was shown that phenylbutyric acid is readily transported in the *Avena* coleoptile (14), the possibility exists that the growth promotion in the apical regions of *Avena* coleoptiles pretreated with phenylbutyric acid, as well as the growth inhibition in the basal regions, are simply the result of different concentrations of phenylbutyric acid in the sections. According to a series of graphs presented by Skoog *et al.* (14, fig. 2), showing the interaction of various concen-

trations of phenylbutyric acid and indoleacetic acid on the elongation of sections of *Avena* coleoptiles, this explanation could be offered as an alternative to Went's which involves a displacement of unknown growth factors moving under the influence of phenylbutyric acid.

The reader will notice that the theory of Skoog *et al.* has much in common with the theories which recently have been proposed to explain the interaction of sulfanilamide and *p*-aminobenzoic acid. According to Woods (87a) and Fildes (87b) the sulfanilamide exerts its bacteriostatic action by competing with *p*-aminobenzoic acid for an enzyme system that is essential for the growth of the susceptible organisms. This theory has found considerable experimental support. Similar cases of competitive action have been reported for pantothenic acid and its analogues and also for tryptophane and some of its analogues. [For discussion see (58, 88, 89).]

Skoog *et al.* (14) presented theoretical considerations concerning the mechanics of the interaction of different compounds with auxin activity, and of compounds chemically related to auxins but inactive as such. They based their considerations on the assumption that auxin acts as a coenzyme and as such has to combine with (*a*) the apoenzyme to form the active auxin complex, which was discussed above, and (*b*) the substrate. This requires that auxins must have two different types of chemical reactivity. Some substances (like phenylbutyric acid) have only the structure to match the apoenzyme, while lacking the structural requirements to react rapidly with the substrate, hence the inhibitory action of phenylbutyric acid. Other compounds will be highly reactive with the substrate but are lacking the structural requirements to combine with the apoenzyme. While finally some auxins have their reactive structures blocked. Although this way of presenting the action and interaction of auxin and auxin-like substances is speculative, it nevertheless makes it possible to visualize, among other things, how a substance can act as an inhibitor and at the same time as an auxin precursor. Such substances exist and are discussed above.

New synthetic growth regulators.—Since the last review on growth regulators was written (1) many more substances have been added to the already imposing list of synthetic growth regulators. Zimmerman & Hitchcock especially have been active in this field (24, 90, 91, 93, 94, 95). Naphthoxy compounds, substituted phenoxy compounds, and substituted benzoic acids are among the substances showing activity on the tomato stem, on root formation, on artificial parthenocarpy,

and on modification of developing plant organs. Although substances such as naphthoxyacetic acid show many of the effects of auxins, such as indoleacetic acid, they do not respond in the *Avena* test (27, 28). Naphthoxyacetic acid, therefore, is not an auxin. Why the substance causes stem curvature in the tomato stem and not in the *Avena* coleoptile is not known at present and should be investigated. It may be that the substance is not readily transported in the *Avena* coleoptile. Such substances have been reported (2). On the other hand it is possible that substances such as naphthoxyacetic acid interact with the native auxins of the tomato stem in a manner such as was discussed in the previous section. The decapitated coleoptiles used in the *Avena* test contain very little native auxin and for this reason might not show these interactions. Gustafson (27) reported activity of 2,4-dichlorophenoxypropionic acid in the *Avena* test. The comparable phenoxyacetic acid had no activity in this test, but it was highly active in inducing negative curvatures in the tomato stem (27, 91, 93). Zimmerman & Hitchcock (93) showed that introduction of the chlorine atom increased the activity of the compound greatly. Phenoxyacetic acid itself has a relatively low activity, *o*-chlorophenoxy acetic acid is twenty times more active, *p*-chlorophenoxyacetic acid is eighty times more active, and 2,4-dichlorophenoxyacetic acid is 650 times more active than the unsubstituted phenoxyacetic acid. The 2,4-dichlorophenoxyacetic acid is active in dilutions as low as indoleacetic acid in causing curvatures of the tomato stem, but the effective range of indoleacetic acid is much wider than that of the phenoxy compound. A compound which is of considerable theoretical interest is 2-bromo-3-nitrobenzoic acid. It is reported to be active on the tomato stem (93); however, Went (personal communication) found it inactive in the *Avena* test.

Interactions between auxin, ethylene chlorohydrin, and ethylene.—Ethylene chlorohydrin is known as an effective agent for breaking dormancy of potatoes (96). The mechanism of this process has been investigated by Michener (97), who found that the apical buds of the potato tuber prevent the lower ones from developing, exactly as in unmodified stems.[4] This apical dominance is destroyed by ethylene

[4] The literature on bud inhibition has been reviewed by Thimann (98). Subsequent experiments supporting a theory of indirect action of auxin (diversion of "calines") were published by Went (99). Experiments on isolated buds supporting the theory of direct action of auxin on the bud appeared in an article by Skoog (100). Snow (101), disagreeing with both, brought forward evidence for the

chlorohydrin treatment. Michener demonstrated that the reason for this is the destruction of auxin in the tuber. When auxin is applied to the apex of the tuber the lost apical dominance can be restored. This is in agreement with the findings of Guthrie (102) and Denny (103, 104) who found that the methyl ester of naphthaleneacetic acid is effective in keeping potatoes in the dormant state.

The possibility exists that this increased auxin destruction is due to an increase in the catalase and peroxidase activity which was reported by Denny, Miller & Guthrie (105, 106) to occur in potato tissue as a result of ethylene chlorohydrin treatment. Similar cases have been reported in dwarf corn (43, 107), dwarf peas (108), and zinc deficiency (109) where an increase in activity of these enzymes has also been correlated with an increased auxin destruction.

Other cases are known which suggest that ethylene and related compounds may lower the auxin level in the plant and thereby cause certain physiological effects. Abscission of leaves, fruits, and flowers has been shown to be under auxin control. Auxin applications will delay or prevent abscission (3, 92, 110). On the other hand ethylene is known as an agent which promotes abscission (112, 113). Just as in the case of the potato tubers, this effect may be due to a lowering of the auxin level by ethylene treatment. Another case of antagonistic action between auxin and ethylene is reported by Cooper (114), who found that the premature induction of flowering by ethylene may be nullified if the treatment is followed by an application of auxin.[5] Certainly, not all effects of ethylene and related compounds can be explained by their effect on the auxin mechanism of the plant, but on the other hand some may be so explained.

Development of galls and nodules.—Virulent strains of *Phytomonas tumefaciens* produce not only galls but in addition the infected plants show all the signs of the presence of large amounts of auxin, such as epinasty, adventitious roots, bud inhibition, cambial activity, and delayed abscission (115). The presence of a high auxin content

presence of an inhibiting hormone. It seems to the reviewer that much of the disagreement might be resolved, if a clearer distinction were made between the inhibition of the bud *per se* and its subsequent growth.

[5] Cooper found that one may distinguish between two periods in the pineapple in regard to flower differentiation. (*a*) About twenty to six weeks prior to normal flower differentiation only ethylene will cause advanced flowering. Auxin applied by itself has no effect, but when applied following ethylene treatment it will nullify the effect of ethylene. (*b*) In a period of not over six weeks prior to normal flowering not only ethylene but also auxin by itself will cause advanced flowering.

of the infected plants is further confirmed by direct auxin analysis. The capacity to increase the auxin concentration in the host plant apparently is not restricted to *Phytomonas,* but it also appears to be one of the earliest symptoms of *Fusarium* wilt (116). It may accompany the invasion of *Rhizobium* (117) and occurs after "invasion" of the style by pollen tubes (118).

Locke, Riker & Duggar (62, 119) also showed that in peptone broth the virulent and the attenuated strain of *Ph. tumefaciens,* as well as *Bacillus radiobacter,* produced equal amounts of auxin. Thus, (*a*) auxin produced by the organism itself could not be responsible for its pathogenicity (119), and (*b*) the auxin found in abundance in plants infected with the virulent organism must be a product of the host plant itself (115).

Locke, Riker & Duggar (115) also reported that a tomato stem which was inoculated with an attenuated strain of *Ph. tumefaciens* could be made to produce fairly large galls when the attenuated gall was treated with lanolin paste containing indoleacetic acid. However, the effect was not very pronounced. Braun & Laskaris (120) had considerably more success, perhaps because they used naphthaleneacetic acid, which they showed to be more effective than indolebutyric or indoleacetic acid. With attenuated strains of *Ph. tumefaciens* they obtained galls on decapitated tomato plants which resembled in many respects those produced by virulent strains. Their technique consisted of applying auxins a few days after inoculation with an attenuated strain. The auxins did not affect the virulence of the attenuated strain. They concluded from their observations that the production of crown galls involves two phases: (*a*) normal host cells are changed to tumor cells, which without further stimulation will not develop into neoplastic growth, and (*b*) stimulation by auxin, which results in the tumor.

According to this reasoning, then, the virulent strain differs from the attenuated one in that the latter lacks the power to induce abnormal auxin formation by the host plant. Perhaps under influence of the virulent strain the equilibrium between auxin precursor and free auxin is shifted to the side of free auxin (see discussion in earlier section of this review).

Cells changed by the crown gall organism into gall cells may have apparently undergone a permanent change. White & Braun (121, 122) showed that tumors which were entirely bacteria-free continued to produce tumor cells exclusively when grown *in vitro* and that frag-

ments of such cultures produced big tumors when transplanted under the bark of plants. It is possible that substances produced by the parasite bring about this permanent change of normal cells into gall cells. Colchicine is an example of a substance capable of changing the genetic make-up of cells (123). Successful experiments of gall production by injection with extracts containing an active principle are rare, but Martin (124) reported that extracts of autoclaved leaf-hoppers and mealy bugs produced galls when injected into meristematic tissues of the stem of sugar cane. A comparison between plant and animal tumors was made by Levine (125).

The role of auxin in the development of *Rhizobium* nodules on roots of legumes may be similar to that in crown galls. Thimann (117, 126) originated the thesis that the nodule arises as a direct result of the production of auxin by *Rhizobium* within the infected root. However, Wilson (127) pointed out that there is no reason to assume that auxin is the cause of root nodule formation, since *Bacillus radiobacter,* which frequently occurs in legume roots, produces *in vitro* even more auxin than *Rhizobium,* yet it never produces nodules. Here also, auxin probably makes cells grow out after they are first changed under the influence of the *Rhizobium.*

Tumors produced at the cut surface of decapitated young plants by the action of relatively high concentrations of auxin have been known for some time. The literature on this subject has been reviewed by Palser (128), who also found that in *Vicia* the epidermis and outer cortical cells did not divide but enlarged, while the inner cortical cells, the endodermis, the cambium, parenchyma of xylem and phloem, the pith, and the rays became active and divided. In this species no roots or root primordia were formed at the swelling which is rather an exception. It was emphasized again that the degree and type of response to a growth substance varies with the species and also with the age of the plant. Polyploidy did occur but Palser believes that since such cells are known to occur spontaneously, they are not the result of indoleacetic acid treatment. The same holds for other cases of polyploidy ascribed to auxin (129 to 132). Polyploidy of *Rhizobium* nodules has been shown to be spontaneous also; the invading bacteria prefer tetraploid to diploid cells, which explains the correlation between *Rhizobium*-infected roots and tetraploidy (127).

Went (87) showed that in pea seedlings no swelling occurs if the roots are removed and suggested that in addition to auxin, substances produced by the roots ("calines") are necessary. However, Beal (77)

found that short stem sections of the bean produced swellings when auxin was applied, provided they were kept on White's nutrient medium. If either sugar or nitrogen was left out of the nutrient, however, no response took place. Isolated sections kept on the complete nutrient medium responded the same as if they were left on the decapitated plant. The wonderful synthetic ability of plants, as compared to animals, was demonstrated by Spoehr (133), who showed that albino maize could be made to grow and produce ears on a diet of sucrose and minerals only. Additions of nutrilites and indoleacetic acid had no effect. The interrelations between sucrose and auxins for elongation in the absence of light was studied by Schneider (134), Bausor (135), and by Went & Bonner (136).

Another type of auxin-induced cell proliferation is that occurring inside ovules. The layer of somatic cells surrounding the embryo sac usually remains one layer of cells in thickness. Cooper & Brink (137) found that in *Nicotiana rustica* this layer would develop into a multilayered growth as the result of pollination with incompatible pollen. This is probably an auxin effect since van Overbeek, Conklin & Blakeslee (138) produced a similar growth from the usually single layered endothelium by injecting auxin into the ovaries of unpollinated *Datura*.

Problems of differentiation.—A case was mentioned above where gall tissue remained undifferentiated (121, 122). White has reported another case (139, 140, 141) where an undifferentiated callus can be made to produce shoots. When tissue of a *Nicotiana glauca* x *N. Langsdorffii* hybrid is cultured on the surface of a semisolid agar medium, the tissue will grow and proliferate but will not show any signs of differentiation. When small callus cultures are completely immersed, growing points start to develop and leaf-like structures complete with stomata and glandular hairs appear. White believes that the oxygen gradient is probably the controlling factor in the differentiation of these callus cultures.

Skoog (141a) investigated the effect of auxin on the differentiation of White's *Nicotiana* callus and found that a concentration of 0.1 to 0.2 mg. per l. of indoleacetic acid added to the liquid medium will inhibit the differentiation. He also found that a relation exists between the auxin concentration effective in inhibiting differentiation and the concentration of the nutrient. When the sucrose concentration was increased from 2 to 4 per cent and the phosphate concentration was quadrupled, the auxin concentrations mentioned above were no

longer found to be inhibitory. However, higher concentrations of auxin still would inhibit.

Another case where differentiation was controlled *in vitro* was reported for *Datura* proembryos (44). These embryos consist of a globular, undifferentiated body only 0.10 to 0.13 mm. in diameter. When grown *in vitro* in a medium containing sucrose and known nutrilites, only a few embryos developed, and these remained undifferentiated. However, when preparations obtained from coconut milk were added to the medium the embryos differentiated perfectly normally and in seven days increased 3,500 times in volume.

Auxin also affects the development of *Datura* embryos in that at concentrations higher than 0.1 mg. per l. it prevents differentiation (142). Embryos which had already differentiated, grew into callus-like structures when placed in a medium containing autoclaved coconut milk (44, 143). The reason for this lies in the high auxin concentration liberated from precursors during autoclaving (44).

Fruit development.—There can be little doubt that auxin is an important factor in the development of the young fruit. It controls the growth of the carpels (144) as well as the growth of the ovules (138). The literature on artificial and natural parthenocarpy has been reviewed by Gustafson (145). As far as the process of natural pollination is concerned it was thought by Gustafson (146, 147) that the initiation of growth of the ovary into a fruit results from the auxin brought into it by the pollen or pollen tubes. However, after it became known (61) that tissues contain considerable amounts of inactive auxin precursor which can be quickly made available as active auxin, van Overbeek, Conklin & Blakeslee (138) came to the conclusion that it is likely that activation of this precursor in the pistil is responsible for part of the fruit development. They calculated that the amount of auxin present in the pollen of an average pollination was insufficient to supply the large amounts of auxin necessary for fruit development and concluded that the pollen provides the pistil with an agent which activates its auxin precursor. This conclusion was confirmed by Muir (118), working in Gustafson's laboratory. He found that very little auxin diffused out of ovaries and styles of unpollinated flowers, but that this amount increased with the time after pollination. The pedicel also gets its auxin from the pollinated ovary which prevents abscission of the young fruit. Extraction of pollen grains and pollen tubes did not indicate them to be the source of the growth hormones.

Murneek & Wittwer (206, 207) reported that parthenocarpic tomato fruits induced by alcohol extracts of immature corn kernels were superior to those obtained by artificial pollination or by the use of synthetic growth substances. It is possible that this may be ascribed to a gradual release of auxin from the large amounts of auxin precursor contained in such extracts. (See *Endosperm precursors,* above.)

It is interesting to note that in fruit development as well as in the development of the crown gall an external agent arouses the "host" to increased auxin production, and thereby causes increased growth, the type of which is determined by the condition of cells of the "host."

Practical Aspects

In this section the reviewer has attempted to group together some papers which have a bearing on more practical problems. In terms of dollars and cents these applications have become of considerable importance. Thus, the

> . . . . hormone spraying of apples is already becoming a standard orchard practice that promises to be of immense value to producers in preventing the dropping of fruit before it has reached good size and color. This single application should save far more than all the money spent on all our hormone research. Yet it was not even dreamed of in the beginning of the research work . . . . (3).

Abscission.—The above quotation by Auchter shows the importance of applications of growth regulators to prevent abscission. Not all plants respond as favorably as apples (4, 148, 149) and not all substances are equally effective (92). Naphthaleneacetic acid or naphthaleneacetamide are equally effective whereas substances such as indoleacetic acid, showed some effect but far less than the first two compounds. It is generally observed that while indoleacetic acid is the most effective of the synthetic substances in the *Avena* test (27) it is far less suitable than naphthaleneacetic acid for treatments of green plants in light (27, 120, 138). The greater stability in the green plant of the naphthalene compound may make it more suitable for general practical use.

Coleus was made the object of detailed studies of the abscission of leaves. Myers (150, 151) came to the conclusion that the delayed abscission of the petioles induced by indoleacetic acid is not so much due to an inhibited development of the abscission layer (which is even present before the leaf is mature) as to a delay in the disintegration of lamellae in the walls of the cells in this layer. Gardner & Cooper

(152) investigating the effectiveness of growth regulating substances found *beta*-naphthoxyacetic acid the most effective (closely followed by naphthaleneacetic acid) when applied as a spray on the leaves. However, methyl indolebutyrate was most effective when the substance was applied as a lanolin paste on the cut surface of the petiole. Lanolin preparations applied to the cut surface of petioles cut to 2 cm. in length were more effective than when applied to petioles 0.5 cm. long. A similar distance effect was observed by Went (99) with auxin-induced bud inhibition of etiolated pea shoots.

The possibility exists that flowers may be kept longer on the plant by application of "hormones" (90), but the application of "hormones" cannot be expected to preserve cut flowers unless bacteria can be prevented from clogging the vessels. The opposite effect, induction of abscission, is of possible practical significance for the perfume industry. Arrillaga & Jones (113) report that ethylene causes abscission of only the ripe petals of ilang-ilang (*Canangium odoratum*) flowers on cut branches. This yields a superior oil uncontaminated by the lower quality oils of the immature petals and ovaries. Another consequence of this effect of ethylene is that storage room operators are cautioned to keep flowers from ethylene producing products such as apples (153).

Fruit set.—After Gustafson (144, 146, 154) had shown how to produce fruits without pollination, newer methods were developed which were more practical than the original lanolin paste treatment. Spray emulsions and even vaporized growth regulators were applied as a means of supplementing the normal processes of pollination and fertilization (25, 155 to 159). The treatments have been outstanding with tomatoes and holly, but conflicting results have been obtained with other species (4). When tomato flowers are treated before pollination has taken place, a high percentage of seedless fruit is obtained, but when treated after pollination most fruits contain seed. Why auxin inhibits fertilization is not known. The germination of pollen and growth of the tube *in vitro* is promoted by auxins in several plants (160, 161). In some instances fertility is promoted by a proper auxin treatment (162), perhaps due to delayed abscission of the style or the whole flower.

Bud inhibition.—When plants are kept in storage it is often desirable to prevent their buds from developing. Methyl esters of naphthaleneacetic acid were successfully employed to prevent shoot development in potatoes (102, 103) as well as in rose bushes (163).

For potatoes the treatment consists of merely storing the tubers in the presence of paper impregnated with the ester, since its vapors are active. Other uses for growth regulators are the delaying of development of flower buds (164) and leaf buds (165) to prevent them from growing out when there is danger of frost damage. This method has also been suggested for prolonging the dormancy of tung flowers where it might be of considerable importance since in two out of five years late spring frosts have seriously reduced the production of tung oil in America (166). In fruit trees it has been found that "hormone" sprays given in the summer will retard the opening of buds the next spring (167).

Rooting.—Root formation under influence of synthetic growth regulating substances was extensively reviewed by Mitchell & Rice (110). It is probably next in economic importance to the "hormone" treatments which delay abscission. For instance, when the main *Derris* supply was lost due to occupation of Far Eastern countries, it became necessary to propagate valuable high-yielding Sumatra clones as rapidly as possible, the largest supply of which is probably present in Puerto Rico (168). Cooper (169, 170) developed a method by which it became possible to utilize for propagation leafy material which had previously been discarded. On leafless *Derris* cuttings the "hormone" treatment was not successful, not because it failed to produce roots, but because the treatment caused severe bud inhibition which prevented the plant from utilizing its roots, and "die back" resulted because of food starvation. The vegetative propagation of the Russian dandelion by Marth & Hamner (171) is another example of the usefulness of growth regulating substances.

Considerable improvement in the technique of applying root-forming substances has been made in recent years. A dust dipping method where talc was used as a carrier of the active chemicals, has been developed (172, 173). A still more convenient method is the alcohol-dip technique in which the basal end of the cutting is dipped in indolebutyric acid or other active chemicals dissolved in 50 per cent alcohol. This method was suggested by Hitchcock & Zimmerman (174) and worked out by Cooper (169, 170) on rotenone-producing plants, for which 2 mg. of indolebutyric acid per cc. is the best concentration.

Another question of practical consideration is the kind of chemical used. Stoutemyer (175) found that about one third of the horticultural plants he tried responded best to "hormones" in the amide form,

another third responded best to the acid itself, and the remaining third responded equally well to both.

Consideration also has been given to substances used for treatment in addition to the usual root-forming substances. Thimann & Poutasse (176) believe that the nitrogen nutrition of the leaf is of great importance for root formation. They showed that in bean cuttings the number of roots is considerably increased by the addition of potassium nitrate (ammonium salts were toxic), adenine, asparagine, and some other organic nitrogenous compounds. Doak (177) found that guanine, serine, and isoleucine when used in addition to naphthaleneacetic acid greatly increased the number of roots on *Rhododendron* cuttings. He suggests that these substances might have the same effect as the "rhizocaline" postulated by Went (87). Favorable responses to sucrose also have been reported again (135).

The condition of the tree from which the cuttings are taken is of considerable importance. Thimann (178) found in conifers, as Gardner had found in apples (179), that the age of the tree (not the age of the branch from which the cutting is taken) is of importance and that side shoots root better than terminal shoots. Individual pine needle bundles rooted more readily than cuttings (180). Hitchcock & Zimmerman (181) found that only the cuttings taken from apple trees before the middle of June responded favorably to indolebutyric acid treatment. A comparable seasonal effect was shown by Gouwentak & Maas (182) to exist for cambial activity induced by auxin. Branches of *Fraxinus* treated in the spring responded to an apical application of indoleacetic acid by producing new wood along the whole length of the test shoot. Branches treated in the fall did not respond. Pearse (183) found that cuttings taken from poorly fertilized plants of *Vitis vinifera* produced more roots (with and without auxin) than cuttings taken from well fed plants. It seems to the reviewer that the high carbohydrate content of the "low salt plants" might be responsible for their better rooting.

An increased root formation due to the action of auxins has been reported for the *Avena* seedling (184), for bean plants—as a result of naphthaleneacetic acid treatment of the tops—(185), for onions (186), and for transplanted pecan trees (187). In the latter case toothpicks impregnated with indolebutyric acid were inserted in the root stubs near the cut ends, which treatment resulted in a much greater development of new roots during the first season after transplanting. The number of roots growing out from the initials of the root band of sugar

cane "seed pieces" (cuttings) was also found to be considerably promoted by relatively high concentrations of naphthaleneacetic acid (188).

General growth promotion.—Many cases have been reported where growth-promoting substances applied to the soil or nutrient solution increased the growth of the plant. This is not necessarily a direct effect on the plant, but it might affect the plant indirectly by affecting the microorganisms of the substrate. Barker & Broyer (189) showed how considerable the effect of soil microorganisms on the growth of plants can be, by comparing the growth of squash plants in sterile waterculture and in similar cultures inoculated with soil. The sterile cultures showed a much better shoot and root growth than the nonsterile ones. Aeration improved the condition of the nonsterile cultures, but the nonaerated sterile cultures were better than aerated inoculated cultures. An effect that might be explained on the basis of keeping growth retarding soil microorganisms down is the growth stimulation due to a fluorescein preparation (Photosensin). Sellei *et al.* (190) observed that most of the dye was found adsorbed on the root system, whereas little or nothing moved up in the shoot. The authors suggest that the fluorescein might affect the "caulocaline" production of the roots; on the other hand an explanation based on suppression of soil microorganisms might be worth looking into.

Some clear-cut cases are known, however, where growth-regulating substances have been applied to plants growing under sterile conditions and thus the effect must be a direct one. Stephenson (191) grew lettuce seedlings for two weeks under aseptic conditions and observed increases of root dry weight of 1,600 per cent and increases of shoot dry weight of 250 per cent over nontreated controls. The treatment was with naphthaleneacetic acid 10 parts per billion. Gorham (192) and Clark & Frahm (193) reported increased growth in *Lemna* grown under sterile conditions when treated with indoleacetic acid.

Hamner (194) reported increased root and top growth in tomato plants when naphthaleneacetamide was given to the nutrient solution. Others, however, reported negative results (195, 196). Laude (197) reported that there was an increase of 13 per cent in the dry weight of bean plants treated with 1 part per billion of indoleacetic acid, provided the potassium level of the nutrient solution was high. Smith *et al.* (198) showed that auxins were effective in inducing root primordia and roots in entire bean plants only when the plants were

grown under conditions which led to a high carbohydrate content. These findings suggest that the disagreement between the various workers in this field might be due to variations in nutrients and other external conditions. The same holds for seed treatments with "hormones": some workers found a favorable effect (199), others no effect (200, 201, 202).

Skoog (109) found that applications of low concentrations of indoleacetic acid to zinc-deficient plants relieved the early symptoms of zinc deficiency somewhat and attributed this to a better utilization of the available zinc. Eaton's (203) observation that indoleacetic acid will to some extent replace boron may be explained in a similar manner. The increased activity of roots under influence of auxins is also reflected in a higher rate of exudation (85). An increased root activity may very well account for the increased growth observed when auxins or related chemicals were added to the substrate.

Some other as yet unknown growth regulators were reported to increase plant growth. Gorham (204) reported in a careful study on the growth of *Spirodela,* that from dung and other sources substances could be extracted by ether or water which are active in promoting the frond number. The effect does not seem to be due to known nutrilites or micronutrient elements. Harper & Cooper (205) reported a similar case with *Cinchona* seedlings. Cow manure extract made the plants appear greener than the controls, yet neither nitrogen applications nor many known nutrilites, auxins, nor amino acids could reproduce the effect.

LITERATURE CITED

1. Went, F. W., *Ann. Rev. Biochem.*, **8**, 521–40 (1939)
2. Went, F. W., and Thimann, K. V., *Phytohormones* (Macmillan, New York, 1937)
3. Auchter, E. C., *Science*, **96**, 283–89 (1942)
4. Gardner, F. E., *Proc. Florida State Hort. Soc.*, **54**, 20–26 (1941)
5. Haagen-Smit, A. J., Leech, W. D., and Bergren, W. R., *Science*, **93**, 624–25 (1941)
6. Haagen-Smit, A. J., Leech, W. D., and Bergren, W. R., *Am. J. Botany*, **29**, 500–6 (1942)
7. Schopfer, W. H., *Plants and Vitamins* (Chronica Botanica, Waltham, Mass., 1943)
8. English, J., Bonner, J., and Haagen-Smit, A. J., *Proc. Natl. Acad. Sci. U.S.*, **25**, 323–29 (1939)
9. van den Honert, T. H., *Verslag. Ver. Proefsta. Personeel*, **13**, 1–18 (1933)
10. Williams, R. J., *A Textbook of Biochemistry* (D. van Nostrand, New York, 1942)
11. Went, F. W., *Am. Scientist*, **31** (3), 189–210 (1943)
12. Went, F. W., *Am. Assoc. Advancement Sci. Pub.*, **14**, 147–58 (1940)
13. Commoner, B., and Thimann, K. V., *J. Gen. Physiol.*, **24**, 279–96 (1941)
14. Skoog, F., Schneider, C. L., and Malan, P., *Am. J. Botany*, **29**, 568–76 (1942)
15. Thimann, K. V., *Collecting Net*, **15**, (4), 66–69 (1940)
16. Williams, R. J., *Science*, **67**, 607–8 (1928)
17. Doudoroff, M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 73–75 (1943)
18. Liebig, G. F., Vanselow, A. P., and Chapman, H. D., *Soil Sci.*, **53**, 341–51 (1942)
19. Went, F. W., *Proc. Koninkl. Akad. Wetenschappen Amsterdam*, **37**, 547–55 (1934)
20. Went, F. W., *Bull. Torrey Botan. Club*, **66**, 391–410 (1939)
21. Thimann, K. V., and Schneider, C. L., *Am. J. Botany*, **26** (10), 792–97 (1939)
22. Schneider, C. L., *Am. J. Botany*, **29** (3), 201–6 (1942)
23. Bausor, S. C., *Am. J. Botany*, **26**, 415–18 (1939)
24. Zimmerman, P. W., and Hitchcock, A. E., *Contrib. Boyce Thompson Inst.*, **12**, 1–14 (1941)
25. Mitchell, J. W., and Whitehead, M. R., *Botan. Gaz.*, **104** (2), 362–65 (1942)
26. Gustafson, F. G., *Proc. Am. Soc. Hort. Sci.*, **40**, 387–89 (1942)
27. Gustafson, F. G., *Am. J. Botany*, **30**, 649–54 (1943)
28. Avery, G. S., Jr., Berger, J., and Shalucha, B., *Botan. Gaz.*, **104**, 281–87 (1942)

29. WILLIAMS, R. J., *Science,* **98,** 386 (1943)
30. BURK, D., AND WINZLER, R., *Science,* **97,** 57–60 (1943)
31. BALL, E. G., *Ann. Rev. Biochem.,* **11,** 1–25 (1942)
32. SKOOG, F., *Cold Spring Harbor Symposia Quant. Biol.,* **10,** 133–34 (1942)
33. WENT, F. W., *Plant Physiol.,* **17** (2), 236–49 (1942)
34. AVERY, G. S., JR., CREIGHTON, H. B., AND SHALUCHA, B., *Am. J. Botany,* **27** (5), 289–300 (1940)
35. AVERY, G. S., JR., BERGER, J., AND SHALUCHA, B., *Am. J. Botany,* **28** (7), 596–607 (1941)
36. AVERY, G. S., JR., CREIGHTON, H. B., AND SHALUCHA, B., *Am. J. Botany,* **28** (6), 498–506 (1941)
37. AVERY, G. S., JR., BERGER, J., AND SHALUCHA, B., *Am. J. Botany,* **29** (8), 612–16 (1942)
38. AVERY, G. S., JR., BERGER, J., AND SHALUCHA, B., *Botan. Gaz.,* **103,** 806–8 (1942)
39. THIMANN, K. V., SKOOG, F., AND BYER, A. C., *Am. J. Botany,* **29** (8), 598–606 (1942)
40. GORDON, S. A., AND WILDMAN, S. G., *J. Biol. Chem.,* **147** (2), 389–98 (1943)
41. KORNMANN, P., *Ber. deut. botan. Ges.,* **53,** 523–27 (1935)
42. VAN OVERBEEK, J., *J. Gen. Physiol.,* **20** (2), 283–309 (1936)
43. VAN OVERBEEK, J., *Plant Physiol.,* **13,** 587–98 (1938)
44. VAN OVERBEEK, J., *Cold Spring Harbor Symposia Quant. Biol.,* **10,** 126–33 (1942)
45. GUSTAFSON, F. G., *Science,* **92,** 266–67 (1940)
46. GUSTAFSON, F. G., *Am. J. Botany,* **28** (10), 947–51 (1941)
47. SKOOG, F., AND THIMANN, K. V., *Science,* **92,** 64 (1940)
48. THIMANN, K. V., AND SKOOG, F., *Am. J. Botany,* **27** (10), 951–60 (1940)
49. WILDMAN, S. G., AND GORDON, S. A., *Proc. Natl. Acad. Sci. U.S.,* **28** (6), 217–28 (1942)
50. LINK, G. K. K., EGGERS, V., AND MOULTON, J. E., *Botan. Gaz.,* **102,** 590–601 (1941)
51. SKOOG, F., *J. Gen. Physiol.,* **20** (3), 311–34 (1937)
52. KRAUS, E. J., *Botan. Gaz.,* **102,** 602–22 (1941)
53. STEWART, W. S., *Botan. Gaz.,* **102,** 801–5 (1941)
54. MITCHELL, J. W., AND STEWART, W. S., *Botan. Gaz.,* **101,** 410–27 (1939)
55. STEWART, W. S., *Botan. Gaz.,* **101,** 91–108 (1939)
56. STEWART, W. S., BERGREN, W., AND REDEMANN, C. E., *Science,* **89,** 185–86 (1939)
57. VAN OVERBEEK, J., *Botan. Gaz.,* **100,** 133–66 (1938)
58. WILLIAMS, R. J., *Ann. Rev. Biochem.,* **12,** 305–52 (1943)
59. THIMANN, K. V., *J. Gen. Physiol.,* **18,** 23–34 (1934)
60. SÖDING, H., *Ber. deut. botan. Ges.,* **53,** 843–46 (1935)

61. van Overbeek, J., *Am. J. Botany,* **28** (1), 1–10 (1941)
62. Locke, S. B., Riker, A. J., and Duggar, B. M., *J. Agr. Research,* **59** (7), 535–40 (1939)
63. van Overbeek, J., *Plant Physiol.,* **15**, 291–99 (1940)
64. van Overbeek, J., and Bonner, J., *Proc. Natl. Acad. Sci. U.S.,* **24** (7), 260–64 (1938)
65. van Overbeek, J., *Proc. Natl. Acad. Sci. U.S.,* **24**, 42–46 (1938)
65a. Berger, J., and Avery, G. S., Jr., *Science,* **98**, 454–55 (1943)
65b. Berger, J., and Avery, G. S., Jr., *Am. J. Botany,* **30**, 297–302 (1943)
66. Sweeney, B. M., *Am. J. Botany,* **28**, 700–2 (1941)
67. Sweeney, B. M., and Thimann, K. V., *J. Gen. Physiol.,* **25**, 841–54 (1942)
68. Sweeney, B. M., *Am. J. Botany* (In press)
69. Northern, H. T., *Botan. Gaz.,* **103**, 668–83 (1942)
70. Commoner, B., Fogel, S., and Muller, W. H., *Am. J. Botany,* **30**, 23–28 (1943)
71. Reinders, D. E., *Proc. Koninkl. Acad. Wetenschappen. Amsterdam,* **41** (7), 820–31 (1938)
72. Stuart, N. W., *Botan. Gaz.,* **100**, 298–311 (1938)
73. Mitchell, J. W., *Botan. Gaz.,* **101**, 688–99 (1940)
74. Clark, H. E., and Kerns, K. R., *Botan. Gaz.,* **104**, 639–44 (1943)
75. Mitchell, J. W., Kraus, E. J., and Whitehead, M. R., *Botan. Gaz.,* **102**, 97–104 (1940)
76. Mitchell, J. W., and Whitehead, M. R., *Botan. Gaz.,* **102**, 393–99 (1940)
77. Beal, J. M., *Botan. Gaz.,* **102**, 366–72 (1940)
78. Bausor, S. C., *Botan. Gaz.,* **104**, 115–21 (1942)
79. Eyster, H. C., *Science,* **97**, 358–59 (1943)
80. van Overbeek, J., *Am. J. Botany* (In press)
81. Meyer, B. S., and Anderson, D. B., *Plant physiology* (D. van Nostrand, New York, 1939)
82. Lyon, C. L., *Plant Physiol.,* **17**, 250–66 (1942)
83. van Overbeek, J., *Am. J. Botany,* **29**, (8), 677–83 (1942)
84. Anderson, D. B., and Kerr, T., *Plant Physiol.,* **18**, (2), 261–69 (1943)
85. Skoog, F., Broyer, T. C., and Grossenbacher, K. A., *Am. J. Botany,* **25**, 749–59 (1938)
86. Went, F. W., *Am. J. Botany,* **26** (7), 505–12 (1939)
87. Went, F. W., *Plant Physiol.,* **13** (1), 55–80 (1938)
87a. Woods, D. D., *Brit. J. Exptl. Path.,* **21**, 74 (1940)
87b. Fildes, P., *Brit. J. Exptl. Path.,* **21**, 67 (1940)
88. Daniels, T. C., *Ann. Rev. Biochem.,* **12**, 447–72 (1943)
89. van Niel, C. B., *Ann. Rev. Biochem.,* **12**, 551–86 (1943)
90. Zimmerman, P. W., *Ind. Eng. Chem.,* **35** (5), 596–601 (1943)

91. Zimmerman, P. W., *Cold Spring Harbor Symposia Quant. Biol.*, **10**, 152–57 (1942)
92. Gardner, F. E., Marth, P. C., and Batjer, L. P., *Proc. Am. Soc. Hort. Sci.*, **37**, 415–28 (1939)
93. Zimmerman, P. W., and Hitchcock, A. E., *Contrib. Boyce Thompson Inst.*, **12**, 321–43 (1942)
94. Zimmerman, P. W., and Hitchcock, A. E., *Contrib. Boyce Thompson Inst.*, **12**, 491–96 (1942)
95. Hitchcock, A. E., and Zimmerman, P. W., *Contrib. Boyce Thompson Inst.*, **12**, 497–507 (1942)
96. Denny, F. E., *Am. J. Botany*, **13**, 118–25 (1926)
97. Michener, H. D., *Am. J. Botany*, **29**, 558–68 (1942)
98. Thimann, K. V., *Biol. Revs. Cambridge Phil. Soc.*, **14**, 314–37 (1939)
99. Went, F. W., *Am. J. Botany*, **26**, 109–17 (1939)
100. Skoog, F., *Am. J. Botany*, **26**, 702–7 (1939)
101. Snow, R., *New Phytologist*, **39** (2), 177–84 (1940)
102. Guthrie, J. D., *Contrib. Boyce Thompson Inst.*, **10**, 325–28 (1939)
103. Denny, F. E., *Contrib. Boyce Thompson Inst.*, **12**, 387–403 (1942)
104. Denny, F. E., Guthrie, J. D., and Thornton, N. C., *Contrib. Boyce Thompson Inst.*, **12**, 253–68 (1942)
105. Miller, L. P., *Contrib. Boyce Thompson Inst.*, **5**, 213–34 (1933)
106. Denny, F. E., Miller, L. P., and Guthrie, J. D., *Am. J. Botany*, **17**, 483–509 (1930)
107. van Overbeek, J., *Proc. Natl. Acad. Sci. U.S.*, **21**, 292–99 (1935)
108. de Haan, I., and Gorter, C. J., *Rec. trav. botan. neerland.*, **33**, 434–46 (1936)
109. Skoog, F., *Am. J. Botany*, **27**, 939–51 (1941)
110. Mitchell, J. W., and Rice, R. R., "Plant-Growth Regulators," *U.S. Dept. Agr., Misc. Pub.*, **495** (1942)
111. La Rue, C. D., *Proc. Natl. Acad. Sci. U.S.*, **22**, 254–59 (1936)
112. Doubt, S. L., *Botan. Gaz.*, **63**, 209–24 (1917)
113. Arrillaga, N. G., and Jones, M. A., *Ann. Rept. Puerto Rico Expt. Sta., U.S. Dept. Agr.* (1943)
114. Cooper, W. C., *Proc. Am. Soc. Hort. Sci.*, **41**, 93–98 (1942)
115. Locke, S. B., Riker, A. J., and Duggar, B. M., *J. Agr. Research*, **57**, 21–40 (1938)
116. Wellman, F. L., *Phytopathology*, **31**, 281–83 (1941)
117. Thimann, K. V., *Trans. 3rd Comm. Intern. Soc. Soil Sci.*, Vol. **A.**, 24–28 (1939)
118. Muir, R. M., *Am. J. Botany*, **29**, 716–20 (1942)
119. Locke, S. B., Riker, A. J., and Duggar, B. M., *J. Agr. Research*, **59**, 519–26 (1939)

120. Braun, A. C., and Laskaris, T., *Proc. Natl. Acad. Sci. U.S.*, **28**, 468–77 (1942)

121. White, P. R., and Braun, A. C., *Cancer Research*, **2**, 597–617 (1942)

122. White, P. R., and Braun, A. C., *Science*, **94**, 239–41 (1941)

123. Dermen, H., *Botan. Rev.*, **6**, 599–635 (1940)

124. Martin, J. P., *Science*, **96**, 39 (1942)

125. Levine, M., *Cold Spring Harbor Symposia Quant. Biol.*, **10**, 70–77 (1942)

126. Thimann, K. V., *Proc. Natl. Acad. Sci. U.S.*, **22**, 511–14 (1936)

127. Wilson, P. W., *The Biochemistry of Symbiotic Nitrogen Fixation* (Madison, Wisconsin, 1940)

128. Palser, B. F., *Botan. Gaz.*, **104**, 243–63 (1942)

129. Greenleaf, W. F., *Science*, **86**, 565–66 (1937)

130. Greenleaf, W. F., *J. Heredity*, **29**, 451–64 (1938)

131. Levan, A., *Hereditas*, **25**, 87–96 (1939)

132. Dermen, H., *J. Heredity*, **32**, 133–38 (1941)

133. Spoehr, H. A., *Plant Physiol.*, **17** (3), 397–410 (1942)

134. Schneider, C. L., *Am. J. Botany*, **25**, 258–70 (1938)

135. Bausor, S. C., *Botan. Gaz.*, **103**, 710–24 (1942)

136. Went, F. W., and Bonner, D. M., *Arch. Biochem.*, **1**, 439–52 (1943)

137. Cooper, D. C., and Brink, R. A., *Genetics*, **25**, 593–617 (1940)

138. van Overbeek, J., Conklin, M. E., and Blakeslee, A. F., *Am. J. Botany*, **28**, 647–56 (1941)

139. White, P. R., *Bull. Torrey Botan. Club*, **66**, 507–13 (1939)

140. White, P. R., *Sigma Xi Quart.*, **30**, 119–36 (1942)

141. White, P. R., *Growth*, **6**, 55–71 (1942)

141a. Skoog, F., *Am. J. Botany*, **31**, 19–24 (1944)

142. Siu, R. G., *Thesis* (Calif. Inst. Technol., Pasadena, 1943)

143. van Overbeek, J., Conklin, M. E., and Blakeslee, A. F., *Am. J. Botany*, **29**, 472–77 (1942)

144. Gustafson, F. G., *Proc. Natl. Acad. Sci. U.S.*, **22**, 628–36 (1936)

145. Gustafson, F. G., *Botan. Rev.*, **8** (9), 599–654 (1942)

146. Gustafson, F. G., *Am. J. Botany*, **26**, 189–94 (1939)

147. Gustafson, F. G., *Am. J. Botany*, **26**, 135–38 (1939)

148. Batjer, L. P., *Southern Seedsman*, 10–28 (February, 1942)

149. Moore, R. C., *Virginia Fruit*, **29** (8), 15–17 (1941)

150. Myers, R. M., *Trans. Illinois State Acad. Sci.*, **33** (2), 89–90 (1940)

151. Myers, R. M., *Botan. Gaz.*, **102**, 323–38 (1940)

152. Gardner, F. E., and Cooper, W. C., *Botan. Gaz.*, **105**, 80–89 (1943)

153. Wright, R. C., Lumsden, D. V., Whiteman, T. M., and Byrnes, J. W., *Cold Storage Ind.*, 149–50 (February, 1941)

154. Gustafson, F. G., *Botan. Gaz.*, **102**, 280–86 (1940)

155. Gardner, F. E., and Marth, P. C., *Botan. Gaz.*, **99**, 184–95 (1937)
156. Gardner, F. E., and Marth, P. C., *Botan. Gaz.*, **101**, 226–29 (1939)
157. Howlett, F. S., *Proc. Am. Soc. Hort. Sci.*, **39**, 217–27 (1940)
158. Howlett, F. S., *Ann. Rept. Vegetable Growers Assoc. Am.*, 203–14 (1941)
159. Zimmerman, P. W., and Hitchcock, A. E., *Contrib. Boyce Thompson Inst.*, **10**, 481–508 (1939)
160. Smith, P. E., *Am. J. Botany*, **29**, 56–66 (1942)
161. Addicott, F. T., *Plant Physiol.*, **18** (2), 270–79 (1943)
162. Eyster, W. H., *Science*, **94**, 144 (1941)
163. Marth, P. C., *Botan. Gaz.*, **104**, 26–49 (1942)
164. Winklepleck, R. L., and McClintock, J. A., *Proc. Am. Soc. Hort. Sci.*, **38**, 94–96 (1940)
165. Mitchell, J. W., and Cullinan, F. P., *Plant Physiol.*, **17**, 16–26 (1942)
166. Sell, H. M., Reuther, W., Fisher, E. G., and Lagasse, F. S., *Botan. Gaz.*, **103**, 788–93 (1942)
167. Hitchcock, A. E., and Zimmerman, P. W., *Proc. Am. Soc. Hort. Sci.*, **42**, 141–45 (1943)
168. Moore, R. H., and Jones, M. A., *Ann. Rept. Puerto Rico Expt. Sta., U.S. Dept. Agr.*, 8–10 (1942)
169. Cooper, W. C., *Ann. Rept. Puerto Rico Expt. Sta., U.S. Dept. Agr.* (1943)
170. Cooper, W. C. (In press)
171. Marth, P. C., and Hamner, C. L., *Botan. Gaz.*, **105**, 35–48 (1943)
172. Grace, N. H., *Can. J. Research, C*, **15**, 538–46 (1937)
173. Stoutemyer, V. T., *Proc. Am. Soc. Hort. Sci.*, **36**, 817–22 (1939)
174. Hitchcock, A. E., and Zimmerman, P. W., *Contrib. Boyce Thompson Inst.*, **11**, 143–60 (1940)
175. Stoutemyer, V. T., *Proc. Am. Soc. Hort. Sci.*, **39**, 253–58 (1941)
176. Thimann, K. V., and Poutasse, E. F., *Plant Physiol.*, **16**, 585–98 (1941)
177. Doak, B. W., *New Zealand J. Sci. Tech.*, **21** (6A), 336A–43A (1940)
178. Thimann, K. V., *J. Franklin Inst.*, **220**, 337–46 (1940)
179. Gardner, F. E., *Proc. Am. Soc. Hort. Sci.*, **26**, 101–4 (1929)
180. Thimann, K. V., and Delisle, A. L., *J. Arnold Arboretum*, **23**, 103–9 (1942)
181. Hitchcock, A. E., and Zimmerman, P. W., *Proc. Am. Soc. Hort Sci.*, **40**, 292–97 (1942)
182. Gouwentak, C. A., and Maas, A. L., *Meded. Landbouwhoogeschool Wageningen*, **44**, 3–16 (1940)
183. Pearse, H. L., *Ann. Botany*, **7**, 123–32 (1943)
184. Kaiser, S., and Albaum, H. G., *Am. J. Botany*, **26**, 749–54 (1939)
185. Kraus, E. J., and Mitchell, J. W., *Botan. Gaz.*, **101**, 204–25 (1939)
186. Levine, M., and Lein, J., *Am. J. Botany*, **28**, 163–68 (1941)

187. ROMBERG, L. D., AND SMITH, C. L., *Proc. Am. Soc. Hort. Sci.*, **36**, 161–70 (1938)

188. VAN OVERBEEK, J., *The Puerto Rico Sugar Manual* (In press, A. B. Gilmore, New Orleans, 1944)

189. BARKER, H. A., AND BROYER, T. C., *Soil Sci.*, **53**, 467–77 (1942)

190. SELLEI, J., SELLEI, H., MAYER, A., AND WENT, F. W., *Am. J. Botany*, **29**, 513–22 (1942)

191. STEPHENSON, R. B., *Plant Physiol.*, **18**, 37–50 (1943)

192. GORHAM, P. R., *Am. J. Botany*, **28**, 98–100 (1941)

193. CLARK, N. A., AND FRAHM, F. E., *Plant Physiol.* **15**, 735–41 (1940)

194. HAMNER, C. L., *Botan. Gaz.*, **103**, 374–85 (1941)

195. HAMNER, M. E., *Botan. Gaz.*, **103**, 576–80 (1942)

196. SWARTZ, D. B., *Botan. Gaz.*, **103**, 366–73 (1942)

197. LAUDE, H. M., *Botan. Gaz.*, **103**, 155–67 (1941)

198. SMITH, O., NASH, L. B., AND DAVIS, G. E., *Botan. Gaz.*, **102**, 206–16 (1940)

199. TANG, P., AND LOO, S., *Am. J. Botany*, **27**, 385–86 (1940)

200. SWARTHEY, J. C., AND CHADWICK, L. C., *Ohio Agr. Expt. Sta. Bimo. Bull.*, **27** (217), 125–44 (1942)

201. STEWART, W. S., AND HAMNER, C. L., *Botan. Gaz.*, **104**, 338–47 (1942)

202. KIESELBACH, T. A., *J. Am. Soc. Agron.* **35**, 321–31 (1943)

203. EATON, F. M., *Botan. Gaz.*, **101**, 700–5 (1940)

204. GORHAM, P. R., *Thesis* (Calif. Inst. Technol., Pasadena, 1943)

205. HARPER, R. E., AND COOPER, W. C., *Ann. Rept. Puerto Rico Expt. Sta., U.S. Dept. Agr.* (1943)

206. MURNEEK, A. E., AND WITTWER, S. H., *Science*, **98**, 384–85 (1943)

207. WITTWER, S. H., *Univ. Missouri Coll. Agr. Research Bull.*, No. **371**, 1–58 (1943)

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BIOCHEMISTRY OF FUNGI

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It has been pointed out in previous reviews (1 to 5) that the diversified biochemical activities of the filamentous fungi are specifically characteristic of the species and strain employed. The production of different organic acids, pigments, antibiotic substances, and growth factors by particular species and strains often differ more strikingly than do the specific morphological characters. Although these fundamental synthetic capacities of different strains must represent inherent potentialities, their expression may be modified by environmental factors, such as the presence of certain trace elements, the temperature, the pH, and aeration.

In the light of recent research showing that certain biochemical activities of the mold *Neurospora* are controlled by specific genes (6) it seems reasonable that similar genetic controls are responsible for many of the varied biochemical activities of fungi. It is felt that the purpose of this review will be best fulfilled if an attempt is made to correlate recent developments in the biochemistry of fungi with this general concept. This approach is perhaps justified, even though in most cases there is as yet no direct genetic evidence. In many instances it is only possible at present to emphasize the diversities and specificities of different strains, while in other instances attempts will be made to interpret the relationships of various products in terms of metabolic reactions, the genetic blocking of which at different points leads to the accumulation of different but sequentially related products.

It is felt that the implications and importance of developments in the biochemistry of fungi are not limited to the fungi. Just as the principles developed from studies of bacterial metabolism and growth-factor requirements have been fruitfully applied to other forms (7, 8, 9), the correlation of biochemistry and genetics which should eventually be possible with the lower fungi should further our understanding of the biochemistry and control of biochemical reactions and specificities in other forms. It is realized that the present approach is not the only one possible, but it is felt that this approach is one of the most important and potentially most fruitful aspects of the biochemistry of fungi.

Vitamin and Growth-Factor Requirements

The rapid accumulation of information regarding the growth-factor requirements of fungi since the subject was last covered in this Review (10) is emphasized by data compiled by Robbins & Kavanagh (11). These authors provide a valuable comprehensive listing by organisms of the growth-factor requirements of the fungi as reported in the literature through 1941. Since then additional reports have further demonstrated the diversities and extreme specificities of the growth-factor requirements of the fungi. The specific vitamin requirements of twelve new strains of a number of species of fungi have been described (12). In general, their requirements are similar to those of previously studied representatives of the same species. The relation of certain fungi to thiamin has been more closely examined. Nineteen fungi are now known which require intact thiamin, fifteen which require both "thiazole" and "pyrimidine," and thirty which require only "pyrimidine"[1] (13). Pyridoxin is essential for *Graphium ulmi* (*Ceratostomella*) (14, 15). Nine species of the wood-destroying *Ceratostomella* have been shown to require different combinations of biotin, pyridoxin, and thiamin (16). Of a group of seventeen fungi examined (17) the various species of *Tricophyton* differed most in their requirements. Five strains grew well without vitamin additions, four required thiamin, and one required both thiamin and inositol. Another, *T. discoides* (18), required thiamin, inositol, and pyridoxin.

Robbins & Kavanagh in their recent review (11) point out that among the fungi, requirements for thiamin and biotin are common, and less frequent for pyridoxin and inositol. None at that time was known which definitely required riboflavin, nicotinic acid, pantothenic acid, pimelic acid, or *p*-aminobenzoic acid of the B-group [see also Peterson (8)]. Although not all of the B-vitamins are thus included in the requirements of strains of fungi found in nature, in view of the importance and functions of the B-vitamins in other forms (19), it might be expected that fungi in general produce and use other members of this vitamin complex. A number of mutant strains of *Neurospora* have been produced by irradiation and found to require certain of these other vitamins which are produced by the normal strain, as shown by biological tests. These include strains which specifically require thiamin, pyridoxin, *p*-aminobenzoic acid (6, 20), nico-

[1] For simplicity, unless otherwise specified, the terms "thiazole" and "pyrimidine" will be used to denote the thiamin intermediates.

tinic acid, pantothenic acid, inositol (21), and choline (22). These findings confirm the predicted importance of these B-vitamins in fungi. It is probable that strains of fungi with similar requirements may in time be found in nature. To the reviewer's knowledge, no yeasts or molds have yet been reported to require riboflavin (8, 11, 17) or folic acid, the remaining two members of the complex. However, riboflavin is synthesized by all yeasts and molds investigated (23 to 30) including *Neurospora* (31).

Although fungi have not been demonstrated to require vitamins or growth factors other than those of the B-group, vitamin C may be synthesized by certain fungi (32), and is reported to stimulate the early stages of growth of several (33). Indole-3-acetic acid is produced by a number of molds, but only in the presence of tryptophane (34). It has been reported to stimulate growth and reproduction of certain fungi (35, 36, 37).

The requirement of *Phycomyces* for two growth substances in addition to thiamin (38) has been partially cleared up by the demonstration that hypoxanthine functions specifically for and is probably identical with the Z_1 factor (39, 40, 41); guanine and a number of other purines are much less active. The chemical nature of the other active fraction has not yet been determined.

The relation of vitamins to growth of fungi may not always be positive and direct. Two instances of inhibiting effects of thiamin have been reported. In one case, the growth of *M.R. atrovirens,* which does not need thiamin supplied to it, is inhibited by the addition of either thiamin or its components (42). Schopfer (43, 44) reported that a similar growth-inhibiting action of thiamin on *Rhizopus suinus* is overcome by inositol which functions as a growth factor. No explanation of these observations is available. (See p. 686 for other examples of inhibitions.)

Vitamins and Respiratory Functions

In analogy with the known functions in other organisms of thiamin, riboflavin, and nicotinic acid as respiratory coenzymes it seems likely that at least these three vitamins act similarly in fungi. This is substantiated by the demonstration of a carboxylase system in *Fusaria* (45), as well as by the partial purification of a glucose oxidase, probably a flavoprotein, from *A. niger, P. glaucum,* or *Citromyces* (46). Evidence has been presented (47) that the heat treatment which the ascospores of *Neurospora tetrasperma* require for germination re-

leases or activates cocarboxylase. Heating *Phycomyces* spores, or treating with pyridine, apparently activates or releases the Z factors (previously mentioned) and permits spore germination (48). The exact role of hypoxanthine (Z_1) is unknown.

The participation of *p*-aminobenzoic acid, pyridoxin, and pantothenic acid in respiratory systems of certain fungi has been indicated in specific strains of *Neurospora*. Each strain, when starved for one of the three vitamins, responds to its addition by a definite increase in Q_{O_2} (49). Much more work remains to be done with fungi in line with recent work with bacteria (50).

The stimulating effect of extracts of *Fusaria* on the respiration of potato tubers (51), the normal host, may involve respiratory functions of certain vitamins, or of unknown substances. Similar relationships may exist between the respiration of wheat plants and infection with parasitic mildews (52, 53, 54).

Genetic Basis of Growth-Factor and Vitamin Requirements

The general development of the concept that the specific growth-factor requirements of microorganisms are due not to intrinsically varied needs but to loss of synthetic powers, has been covered in detail many times (55, 56). One of the main contributions of this concept is that the losses in synthetic capacities are frequently correlated with an adaptation or modification to more and more complex environments as far as vitamin supplies are concerned. The observations on fungi support this concept, since it may be said that most parasitic or pathogenic fungi have absolute deficiencies for growth factors. Following the enunciation of these general principles it has been repeatedly shown, by means of a variety of techniques, that in every case so far examined, including fungi (10, 30, 57, 58, 59), an organism which does not require an external source of any one of the B-vitamins can synthesize it from simpler constituents. It has also been shown (13, 57) that specific vitamin deficiencies of various organisms or strains of organisms may result from the blocking of different steps in the synthesis of the vitamin. For example, thiamin may not be synthesized because of the loss of capacity to produce "thiazole" or "pyrimidine," to combine these two components into thiamin, or because of losses of several of these capacities.

The specificity of these synthetic abilities and the correlation of their losses with an evolutionary sequence, suggests by analogy with other biochemical reactions which are known to be under a more or

less direct genetic control (60 to 63) that the syntheses of these vitamins should also be under genetic control. The observed losses in synthetic abilities should then be directly correlated with gene mutations. Gene mutations to the recessive, relatively less active, form are most common and in vitamin synthesis evolutionary changes are generally toward losses in capacities.

In the case of bacteria, most fungi, higher plants, and animals, there are as yet no means of directly testing this general hypothesis. However, the genetics of the mold *Neurospora* is well known (64, 65) and a number of mutations affecting morphological characters have been studied in this organism. With this fungus it has been established that the synthesis of vitamins and amino acids is gene-controlled (6, 21, 66). A number of distinct mutant strains of *Neurospora* have been produced in which the synthesis of various vitamins is deficient. In most cases the mutant character is inherited as if it were associated with the mutation of a single gene. Apparently the mutation of each gene causes a specific biochemical defect (see p. 674 for examples). In regard to thiamin synthesis, Robbins & Ma (13) conclude that in fungi occurring in nature the loss of the capacity for "pyrimidine" synthesis is most frequent. Only one (*Mucor ramannianus*) has lost only the ability to make "thiazole." None is known in nature which can synthesize the two components but at the same time cannot put them together. Most fungi have lost more than this one synthetic capacity. However, the production of a "thiazole"-deficient mutant of *Neurospora*, and of a "thiaminless" mutant[2] (21), which probably can make the components, supports the conception of analogous losses in synthetic ability in all fungi as being the result of separate, perhaps consecutive, gene mutations.

The significance of these results is not limited to the synthesis of vitamins and amino acids but also applies to the general relations of genetics to all biosynthesis, to metabolism in general, and to the problems of cellular growth and differentiation (21, 62). The study of specific biochemical reactions in such genetically altered forms is analogous to the use of chemical inhibitors in studying metabolism. Since gene mutations are so much more specific in their action than chemical inhibitors, the results obtained should be correspondingly more reliable.

[2] Mutant strains of *Neurospora* are designated by adding the suffix "less" to the name of the substance for which the particular strain is deficient, i.e., "nicotinicless"—a strain deficient in nicotinic acid, which therefore requires for growth the addition of nicotinic acid.

Genetic Control of Other Biochemical Reactions

Genes may be assumed to control specific biochemical reactions through their primary control of the production or specificity of enzymes. Winge & Laustsen (67) first suggested the genetic control of specific enzymes in yeast. In hybrids between yeast strains with different fermentative capacities the fermentations of raffinose, mellibiose, and sucrose were found to be controlled by dominant genes. When only the recessive mutant forms of the genes were present in the hybrid, fermentation did not take place. This was attributed to absence of specific enzymes. The inability of certain strains of bacteria (*E. coli*) to ferment lactose has been attributed to impermeability of the cells to the disaccharide (68). However, the original interpretation of the results with yeast is supported by demonstrations of analogous genetic control of certain enzymes in plants and animals (69, 70). Further work with yeast (71) has led to the suggestion that wild and cultivated yeast strains may differ primarily in genes which modify the production or activity of enzymes concerned in fermentation reactions. The vitamin requirements of certain yeasts may be associated with the production of certain fermentation enzymes, although probably not in a causal relation. It has been found that a number of lactose-fermenting yeasts require nicotinic acid for growth, while other non-lactose fermenting strains do not need an outside supply of this vitamin (72). This might suggest the separate occurrences of independent gene mutations involving the two separate characters. The production of enzymes involved in amino acid oxidations may vary in different strains of *Allomyces* (73). The production of permanently altered cyanide-insensitive strains of yeast should be recalled (74). This probably is an induced deficiency in the cytochrome oxidase or other cyanide-sensitive enzyme systems. The amounts of B vitamins synthesized by different yeast strains derived by selection vary considerably (25). Similar variations in the synthesis of riboflavin by yeast strains found in nature have been attributed to genetic differences (26). The amount of this vitamin synthesized by a yeast (*Candida guillermondia*) is markedly affected by cultural conditions (75).

The ability to synthesize particular vitamins may be redeveloped, perhaps through mutation and selection, as indicated in yeast for eight vitamins (25) and in *Fusarium avenaceum* for biotin (76). Such instances are reminiscent of "training" in bacteria (9, 56, 77). These

and other similar variations in bacteria (78, 79) may have a genetic basis, as has been suggested in the development of phage-resistant strains of *E. coli* (80).

There are other cases in which particular properties or characters of fungi appear to be gene-controlled. Many mutant characters which affect phytopathogenic smut and rust fungi are known (81, 82). In tomato-wilt *Fusaria,* the toxicity for tomato plants of extracts of isolated mutant or variant strains (83, 84) is correlated with the virulence of the strain (85). Pathogenic differences in certain rust fungi are not correlated with their requirements for thiamin, riboflavin, or for nicotinic, ascorbic, or indole-3-acetic acids (86).

An interesting case in fungi is the genetic control of luminosity in *Panus stypticus* (87). A single gene difference was demonstrated between a luminous American strain and a non-luminous European strain. In hybrids the positively acting gene (for luminosity) is dominant over its allele.

Sexual Phases and Biochemical Reactions

Genetic control of biochemical reactions in fungi in other cases is suggested by the association of a given reaction with mating type or sexual phase (which are gene-controlled), or by the induction of sexual spore formation by definite chemical compounds. The difference in the production of fumaric acid by strains of *Rhizopus nigricans* of opposite mating types (88) indicates a genetic control of metabolism, perhaps indirect. The nature of the growth factors essential for spore formation in fungi has been previously reviewed (10, 58). Perithecium formation by *Microsporum audouini* (an imperfect fungus) has been observed only in the presence of unknown substances produced by a certain bacillus (89). Different nuclear phases of *Ustilago nuda* apparently vary in their thiamin requirements (90).

The stimulating effect of growth of *A. niger* or of *A. niger* culture filtrates on conjugation in *Zygosaccharomyces* has been shown to depend on at least two extractable substances (91). *Zygosaccharomyces* may represent genetic stages in the life cycle of *Saccharomyces* (92). If this were true, the inability of members of the genus *Zygosaccharomyces* to conjugate normally and form viable spores might be correlated with the genetically determined concentration level of specific conjugation factors. A partial replacement of the conjugation factors for *Zygosaccharomyces* with glutaric acid in combination with riboflavin has been demonstrated, although these are probably not the active sub-

stances produced by *A. niger* (93). It seems significant that dicarboxylic acids are also involved in sexual processes in the water-mold *Achlya*, in plant wound healing (94), in the excystment of protozoa (95), and in respiration and growth of *Neurospora* (96).

Raper (97) has shown that the sexual processes in the Phycomycete, *Achlya*, are controlled by sequential production of four diffusible substances or "hormones." "Hormone A," the initial substance in the sequence of reactions, can be replaced by certain dicarboxylic acids, or by certain barbituric acid derivatives, but is probably a ketone (98). The action of *Achlya* hormones may be somewhat analogous to the roles of crocin and related compounds [see discussion by Schopfer (58)] in the development of motility and in conjugation of *Chlamydomonas eugametos*, in which the biochemical reactions are definitely controlled by specific genes (99). It is of interest in this regard that the rare γ-carotene found in the water-mold *Allomyces* is the only carotene present in most species, and is restricted to the male reproductive cells (100).

Induction of Mutations or Variants in Fungi

A number of investigators have attempted to use with fungi techniques by which bacterial "variants" or "mutants" have been obtained. The identification of a given change induced by physical or chemical treatments as a true gene mutation is possible only when genetic tests can be made. So far this has been done only with *Neurospora*, in which both morphological and biochemical mutant strains have been produced.

In view of the early beginning of biochemical investigations with *Aspergillus niger* it is not surprising that attempts have been made to induce mutant strains in this fungus. Treatment with radium (101) has been reported to produce races which differ morphologically and biochemically in acid production and amylolytic capacity. Distinctive morphological strains of *A. niger* have been produced by treatment with reagents such as hydrogen peroxide, ninhydrin, and nitrite (102). On the basis of these results, it has been suggested that these treatments change the enzymatic complements of the strains, and thereby alter their morphological characters. Since growth of the "mutant" strains on certain media which contained lysine in addition to other amino acids led most quickly to reversion to the original state, it has been suggested that the loss or recovery of ε-amino groups from lysine units of the cell proteins may be one factor involved in this mutation

and reversion (103). It is exceedingly difficult to evaluate this work at present, since it is impossible to determine whether or not these mutant strains actually represent gene mutations. In a similarly uncertain category are modifications induced in yeast strains by various treatments, hydrogen cyanide (74), camphor (104), and, as with higher plants, colchicine and acenaphthene (105). Strains of *Staphylococcus aureus* have been isolated from cultures grown in the presence of sulfanilamide, which owe their increased resistance to sulfanilamide to an increased *p*-aminobenzoic acid synthesis, not dependent on the continued presence of sulfanilamide (106).

Symbiosis and Related Problems

The phenomenon of symbiosis between different organisms or species has been known for some time. Since the recognition of the differences in growth-factor and vitamin requirements of various organisms, one satisfactory explanation of the phenomenon, based on the vitamin requirements of the symbionts, has been developed (57, 58, 107). Two organisms with different synthetic abilities and therefore different requirements, by exchanging the vitamins they can make, can grow together in media in which neither could grow separately. Successful artificial symbiotic cultures of fungi have been obtained in which *Polyporus adustus* supplies biotin and *Nematospora gossypii* supplies thiamin (108). Another instance in which each symbiont supplies different portions of the thiamin molecule (*Mucor ramannianus* and *Rhodoturula rubra*) has been studied (109, 110). This symbiosis requires the presence of certain inorganic cations (111). More recently a number of successful symbiotic cultures of *Ceratostomella* species have been produced (16). These depend on the reciprocal exchange of thiamin and pyridoxin. The production of such symbiotic cultures constitutes one conclusive method of proving that an organism can synthesize a particular vitamin or portion of a vitamin molecule.

The significance of another related problem has not been fully realized in the past. This is the phenomenon of heterocaryosis, which is of wide-spread occurrence in fungi (112, 113) and perhaps in other organisms (114). Heterocaryosis in fungi involves the fusion of hyphae of two genetically different strains with the production of cells which then contain genetically heterogeneous nuclei. Dodge (115) has produced such heterocaryotic cultures experimentally, and has shown that the resulting hyphae containing the nuclei of two different slow-growing forms of *Neurospora tetrasperma* grow much more rapidly and

vigorously. Similar heterocaryons have been made between biochemical and morphological mutant strains of *Neurospora crassa* and can be used as tests for gene dominance, in a manner analogous to dominance studies in diploid cells of most plants and animals (21). It has been shown that heterocaryosis is a general phenomenon in *Neurospora* and quantitative methods of studying dominance relations in *Neurospora* have been developed (116). These methods should be of great value in accurately measuring the contributions of genes which govern the synthesis of vitamins, amino acids, and other substances. Beadle & Coonradt (116) also discuss the use of heterocaryons in tests for allelism, as well as some further points of general biological significance, including the analogy of the physiological or biochemical bases of heterocaryosis and heterosis, the value to a haploid heterothallic organism of this phenomenon in evolutionary competition, and the possible role of heterocaryosis in the evolution of sexual reproduction.

Biosynthesis of Vitamins, Growth Factors, and Amino Acids

One of the fundamental problems which confront the biochemist is the question of the biosynthesis of cell constituents in general and of the vitamins and other growth factors in particular. Because of the diversities in the synthetic abilities of the lower fungi, these organisms have already proven of value in a clearer understanding of the chemistry of biosynthesis of certain of the vitamins and other growth factors. Any biosynthesis must be viewed as a consecutive series, or converging parallel series, of biochemical reactions. A given synthesis in an organism may be blocked in one or more particular reactions. By the study and comparison of the synthetic abilities of different organisms with regard to a particular substance, the biochemical steps in the synthesis of this substance can be determined. Instead of comparing different organisms which may vary in several respects, there are a number of advantages in comparing different strains of the same organism, especially if these vary in only a single reaction, as do the mutant strains of *Neurospora*.

Thiamin.—Investigations of the synthesis and destruction of thiamin by fungi have indicated that this vitamin is continually being synthesized and broken down, that it may normally be in a state of "dynamic equilibrium" perhaps analogous to that of other cell constituents, proteins and amino acids (117), and perhaps purines and pyrimidines (118). The greater effect on growth of *Phycomyces* of an excess of "thiazole" compared to an excess of "pyrimidine" (119,

120) has led to the view that the thiazole portion is attacked only in the intact vitamin molecule by *Phycomyces* (119). Inactive vitamin analogues are also broken down, and the liberated vitamin-pyrimidine thus made available for resynthesis of thiamin (119, 120, 121). Reinvestigation of the synthesis and breakdown of thiamin and its components by *Phycomyces* and in addition by *Phytophthora, Mucor ramannianus,* and *Sclerotium rolfsii* has supported these concepts and indicated that thiazole destruction and thiamin synthesis may be correlated (122).

Relatively little is known regarding the synthesis of the thiamin components in fungi. A combination of the intermediates in the chemical synthesis of "thiazole," thioformamide, and chloracetopropylalcohol, which can replace "thiazole" for isolated pea roots, apparently cannot be synthesized into "thiazole" by *Phycomyces* (123). The syntheses of "thiazole" by *Phycomyces* and the pea root must therefore either take place along different pathways or the synthesis in *Phycomyces* is blocked after the synthesis of these two precursors.

Various fungi have long been recognized to differ in their ability to synthesize thiamin from its components (13, 57, 58). *Phycomyces blakesleeanus* is the classical example of a mold which can perform this synthesis, while *Phytophthora cinnamomi* has been assumed to be deficient in this ability, as well as being unable to synthesize "thiazole" and "pyrimidine" (124). Recent work (125) has shown that *Phytophthora* cannot carry out the final step in the synthesis of thiamin, presumably because it lacks a highly stable factor of unknown nature. This "Factor S" has been found by Kidder & Dewey in plant extracts which have been freed of thiamin and its intermediates by treatment with hot alkali and ultraviolet light. The results suggest a general significance of Factor S, since it seemed to be richest in green leaves of plants where thiamin is synthesized and very low in animal tissues and products. *Phytophthora* requires both intermediates as well as Factor S for thiamin synthesis, and the absence of Factor S must be independent of the need for the two intermediates, since two protozoa (*Tetrahymena gelii* and *T. vorax*) were shown to be deficient only in Factor S, and since *Phycomyces* presumably can supply its own Factor S.

Pyridoxin.—It has been suggested that the synthesis of pyridoxin by certain lactic acid bacteria involves alanine as an essential intermediate (126). The nature of a more active pseudopyridoxin (127) and its physiological relation to pyridoxin remains in question, al-

though it seems possible that pseudopyridoxin is a decomposition product of pyridoxin itself, since pyridoxin may be changed to "pseudopyridoxin" by autoclaving with any of the following substances: cystine, glycine, ammonia, or thioglycollic acid (128), and by treatment with hydrogen peroxide (129). The response to pseudopyridoxin and the ability to use alanine for the synthesis of pyridoxin apparently are not related since in *Lactobacillus casei* alanine cannot replace pyridoxin (126) although this bacterium apparently responds to pseudopyridoxin (128). Neither alanine nor pseudopyridoxin seems to be involved in the biosynthesis or action of pyridoxin in the fungi so far tested, as shown by investigations with six strains of pyridoxin-requiring *Ceratostomella* (130) and with a "pyridoxinless" mutant of *Neurospora sitophila* (131). Of a number of organisms so far tested, including yeast, fungi, and rats, only certain lactic acid bacteria respond better to pseudopyridoxin (and may even fail to respond to pyridoxin). This makes it unlikely that pseudopyridoxin is of general significance.

Pantothenic acid. — A "pantothenicless" mutant of *Neurospora crassa* (21) is the only filamentous fungus known which has an absolute requirement for exogenous pantothenic acid. This strain requires intact pantothenic acid, and presumably is able to synthesize β-alanine and pantoyl-lactone, but is unable to complete the synthesis. The mutant may lack some component of an enzyme system comparable to that demonstrated in yeast (132, 133) in which β-alanine and pantoyl-lactone seem to be normal precursors of pantothenic acid. It has been indicated that salicylic acid may inhibit the biosynthesis of pantothenic acid in bacteria, as shown by its effect in making strains of *Corynebacterium diphtheriae* sensitive to pantoyl-taurine (134), or by preventing the growth of bacteria normally able to synthesize pantothenic acid, possibly by competitively inhibiting a suggested conversion of α,γ-dihydroxyisocaproic acid to pantoyl-lactone (135).

Biotin.—Evidence has been presented which leaves little doubt that pimelic acid and cystine are precursors in the biosynthesis of biotin by *C. diphtheriae* (136) and by *A. niger* (137). However, in other organisms such as *L. casei* (138) and *Torula cremoris* pimelic acid cannot replace biotin, although aspartic acid may partially do so for *Torula cremoris* (139). It seems likely that in these microorganisms as well as in a number of fungi, including strains of *Ceratostomella, Grossmannia, Fusaria, Neurospora,* and *Nematospora* (140), the biosynthesis of biotin is blocked at stages later than that at which

pimelic acid and cysteine are effective. The biosynthesis of biotin from desthiobiotin by *S. cerevisiae* has recently been demonstrated (141). Desthiobiotin can also replace biotin for a number of microorganisms including *Saccharomyces, Zygosaccharomyces, Neurospora,* and certain strains of *Ceratostomella* (142). This compound, which may therefore be a normal precursor of biotin, not only has no biotin activity but is antagonistic to exogenous biotin for *L. casei* (141, 142), *Sordaria fimicola,* and *Ceratostomella pini 416* (142).

p-Aminobenzoic acid.—Studies of a "*p*-aminobenzoicless" mutant strain of *Neurospora crassa* (6) have suggested that in this fungus the action of sulfanilamide is through an inhibition of the utilization of *p*-aminobenzoic acid (p.a.b.) and not of its synthesis (20). This is in agreement with the accepted views on the competitive nature of growth inhibition by this and similar chemotherapeutic agents (143). The use of this mutant strain of *Neurospora* has also made it possible to dissociate the effect of pH on sulfanilamide activity from its effect on p.a.b. activity, and to show that the availability of the vitamin to *N. crassa* becomes greater as the pH is lowered (144). This increased availability of the molecular form of p.a.b. may be simply a question of uptake by the mold, since the molecular forms of most substances more readily pass through cell membranes (145). The biosynthesis of p.a.b. by *Neurospora* has been investigated (20) with the use of both wild type and mutant strains. A number of related compounds and theoretically possible precursors had some growth-promoting activity. However, the absolute activity was so low in comparison with that of p.a.b. that it was concluded that these compounds, which included aniline, *p*-nitrobenzoic acid, *p*-toluidine, and *p*-dimethylaminobenzaldehyde, are probably not normal intermediates in the biosynthesis of p.a.b. It now seems likely that some or all of these compounds were active *per se*. It has been shown (146) that certain derivatives of p.a.b. which should not be easily converted to p.a.b., such as the 2-fluoro, the 2-bromo, the 2-iodo, and the 3-carboxy derivatives, are definitely active for the *Neurospora* mutant and for *Cl. acetobutylicum,* and act as sulfanilamide antagonists for *E. coli.* 2-Chloro-*p*-aminobenzoic acid, on the contrary, has sulfanilamide-like activity for *E. coli,* which does not require exogenous p.a.b. for growth. Since compounds without an aromatic nitrogen (tyrosine, benzoic, and *p*-hydroxybenzoic acids) had no activity for the *Neurospora* mutant and did not increase p.a.b. synthesis by wild-type *Neurospora* [compare the effect of pimelic acid on biotin synthesis by *A. niger* (137)], it was concluded

(20) that the biosynthesis of p.a.b. in *Neurospora* probably involves the introduction of nitrogen into an aliphatic compound prior to the formation of the aromatic ring.

Choline.—The synthesis of choline and its functions are probably similar in fungi and in animals. Investigations of a "cholineless" mutant of *Neurospora* (22) have shown that one probable function of choline is the formation of lecithin, which is also active. Another probable function is that of a source of labile methyl groups, since methionine has a sparing action on choline for the *Neurospora* mutant, but cannot replace choline. In animals, ethanolamine can be converted to choline by methylation. [See Borsook & Dubnoff (147) for a summary of recent work.] The synthesis of choline by the *Neurospora* mutant may be blocked in this methylation, since the methyl group of methionine cannot be used in choline synthesis and since ethanolamine is inactive for the "cholineless" mutant (22).

Pyrimidines. — Results obtained with two different pyrimidine-requiring mutants of *N. crassa* have shown (148) that uracil and cytosine nucleosides have from 10 to 60 times greater activity for these strains than free uracil. Loring & Pierce (148) point out the similarly greater activity of nucleosides in animal nutrition, and suggest that nucleosides may also be more active for other microorganisms which require pyrimidines (149, 150). Since cytosine was inactive, while both cytidine and cytidylic acid were active, it was concluded (148) that cytosine is probably not a normal intermediate in the synthesis of the corresponding pyrimidine nucleoside or nucleotide. These results may indicate also that cytosine is not concerned in the normal synthesis of uracil. Presumably the deamination of cytosine can take place only in the nucleoside or nucleotide. The greater activity of the nucleosides compared to the nucleotides may be a question of rate of absorption.

Amino acids.—Most fungi can synthesize their amino acids from inorganic nitrogen, as demonstrated by the isolation of most of the known amino acids from mycelial proteins (151, 152, 153). Methionine and cystine may be present in certain fungi in low concentrations (154). With most fungi investigated, however, growth is improved by the addition of amino acids, which indicates that the rate of amino acid synthesis may be limiting (11, 155), as with many bacteria. Attention was first drawn to the similarities in the amino acid requirements of the rat and of bacteria by Fildes & Richardson (156) and by Mueller & Kapnick (157). Recent investigations have in general sup-

ported the analogies. Most amino acids which cannot be synthesized, and exogenous sources of which are therefore required, are characterized by having either rings or branched chains, or organic sulfur. However, exceptions to this generalization are known [for earlier references see (77)], for certain bacteria may require glycine, aspartic, and glutamic acids, or even glutamine (158, 159, 160). All the mutant strains of *Neurospora crassa* which have been studied are characterized by their inability to synthesize particular amino acids, all of which with the exception of proline are also amino acids essential for the rat. It is of interest that of the amino acids so far investigated by nitrogen-balance experiments in humans, threonine, leucine, isoleucine, and phenylalanine are essential, as they are also for growth of the rat, while histidine is not (161). Only one amino acid not essential for the rat, viz. proline, is required by a mutant strain of *Neurospora* (162).

Since the syntheses of amino acids by the mutant strains of *Neurospora* are probably blocked in single reactions which in most cases lead to deficiencies in single specific amino acids (21), at least the final steps in the synthesis of each of these amino acids must be characteristic and different. The direct genetic control of these syntheses in *Neurospora* therefore suggests that deficiencies in other forms for each of these amino acids may be independent and may be due to analogous specific gene mutations. According to this general concept, deficiencies for the other "nonessential" amino acids are rare or unknown for one or both of the following reasons. Their synthesis may involve reactions common to the synthesis of other biologically essential substances so that a mutation would have a lethal effect, or there may be a number of different synthetic paths for each of these amino acids so that a number of mutations would be required to block its synthesis. These nonessential amino acids would also have in common their easy and relatively direct formation from sugar and sugar degradation products.

With the exception of alanine, and the dicarboxylic amino acids, there is rather little information available on the early reactions in the formation of amino acid carbon skeletons from sugars. Studies on the utilization of amino acids as carbon and as nitrogen sources by *A. niger* (163, 164) have led to the view that those amino acids in which the nitrogen was as available as ammonium nitrogen and in which the availability of carbon approached that of sugar carbon, constituted a group of primary amino acids formed fairly directly from

sugar. These amino acids included no branched chain or aromatic amino acids. In these experiments a mixture of proline, glutamic acid, and ornithine was utilized almost as well as a source of both carbon and nitrogen as sucrose and ammonium salts. Somewhat similar experiments with *Endomyces vernalis* (165) have indicated that in this yeast added lactic and pyruvic acids and acetaldehyde do not increase protein synthesis, and that glycine, aspartic acid, and alanine, classed by Steinberg as primary amino acids, do not serve as carbon sources. Interpretations of such results are questionable, since they are based on the unproved premise that amino acids are formed from and reconverted to sugars by the same biochemical pathway.

As mentioned above, most amino acid mutant strains of *Neurospora crassa* specifically require only one amino acid and the genetic block in these cases is therefore in a reaction specific to the biosynthesis of only one amino acid. Studies on a number of these mutant strains have provided information as to the mechanisms of certain biosyntheses and as to the nature of the genetically blocked reactions. The results confirm the expectation (21) that different genes control different biochemical reactions and that the number of different mutants affecting a given synthesis is a measure of the number of biochemical steps directly involved in the synthesis.

One mutant strain of *Neurospora crassa* has been found to require the two amino acids isoleucine and valine in an optimal ratio of 70 to 80 per cent valine and 30 to 20 per cent isoleucine (166). An inadequate supply of valine can be supplemented by leucine. Since this strain presumably is blocked only in one step, the syntheses of isoleucine and valine must involve either a common precursor or a common reaction. The activity of leucine under appropriate conditions indicates either a partial replacement of valine by leucine or an interconversion of these two amino acids. Certain observations with bacteria also suggest interrelations between these three amino acids (167, 168).

Arginine is another amino acid the biogenesis of which has been studied in fungi. Srb & Horowitz (169) have investigated fifteen mutant strains of *N. crassa* and have shown that the synthesis of arginine in *Neurospora* involves an ornithine cycle similar to that demonstrated in mammalian liver (170). [See Borsook & Dubnoff (147).] Seven genetically different mutant strains have been shown to be unable to carry out certain steps in the synthesis. One strain cannot convert citrulline to arginine, two are unable to convert ornithine to

citrulline, and four cannot synthesize ornithine. Since the two which are unable to make citrulline from ornithine are genetically different, two steps in this conversion are indicated on theoretical grounds. Similar reasoning suggests at least four steps in the synthesis of ornithine. The cyclic nature of these reactions in the wild type strain was proved by the demonstration of the presence of arginase and urease and by the isolation of ornithine formed from added arginine by *Neurospora* arginase. This is the first demonstration of the functioning of the ornithine cycle in plants, and may explain the role of urease in plants if this cycle is of general occurrence, as seems likely.

The biosynthesis of tryptophane in *Neurospora crassa* has been found (171) to involve a reaction in which indole and serine are condensed, apparently by an intramolecular dehydration perhaps analogous to the intermolecular dehydration of serine by *E. coli* (172). It was shown that only *l*-serine was active, and that the product (isolated as acetyl-*dl*-tryptophane) was *l*-tryptophane. Evidence was presented that a similar reaction is involved in the synthesis of tryptophane by *E. coli,* and that the production of indole by this bacterium takes place through a reversal of the condensation reaction rather than by the scheme proposed by Krebs *et al.* (173). The participation of serine in the synthesis and breakdown of tryptophane adds another biological reaction of serine to those described by Binkley & du Vigneaud (174), Stetten (175), and Chargaff & Sprinson (172). The synthesis of tryptophane in one mutant strain of *Neurospora* has been found to be blocked before anthranilic acid, and, in another, between anthranilic acid and indole (176). Anthranilic acid was isolated as a product of the mutant which is unable to convert it to indole. These results, together with the inactivity of theoretically possible intermediates between indole and tryptophane prove that in *Neurospora* tryptophane is synthesized by way of anthranilic acid and indole, as is also probable with certain bacteria (177, 178), and by the conversion of indole to tryptophane in a single step (171).

The available evidence indicates that the biosynthesis of many amino acids proceeds by oxidation of the alpha hydroxy acid and by amination of the keto acid. However, evidence is accumulating which suggests that this may be true only for the primary amino acids which can be formed directly from sugar degradation products; the keto acid analogues of valine (166) and of leucine (179) are fully equivalent to the amino acids for the appropriate mutant strains of *Neurospora.* However, the utilization of the two hydroxy acid analogues seems to

require a preliminary period of "adaptation" (see p. 685) and therefore the hydroxy acid analogues may not be normal intermediates in these syntheses (161). Keto acids might be formed directly from unsaturated acids, perhaps by reactions analogous to those suggested for the oxidation of crotonic acid (180). In the mutant which requires both valine and isoleucine a mixture of the two hydroxy or keto acids is inactive. However, either keto acid is active in the presence of the other amino acid. Since only one reaction should theoretically be blocked in this mutant it has been suggested that the synthesis of the two amino acids valine and isoleucine may not normally involve the keto acids (166). Tryptophane is synthesized from indole and serine by *Neurospora* and probably by *E. coli,* and indolepyruvic acid is inactive for *Neurospora* (171). The direct amination of the keto acid analogue is therefore not a normal step in this biosynthesis. The ability of *E. coli* to produce tryptophane from the keto acid (181) may have no relation to the normal biosynthesis. Altogether there is now good evidence that a number of amino acids can be synthesized biologically by reactions other than the direct reductive amination of the keto acid analogues—that is, by reactions in which the amino group is introduced prior to the final step in the synthesis. These amino acids include tyrosine (182), aspartic acid (183), cystine (174, 175), tryptophane (171), arginine (169, 170), possibly serine and threonine (172), and perhaps isoleucine and valine (166). Although the rat can aminate the keto acid analogues of all the amino acids tested except lysine, it cannot synthesize any of the essential amino acids from simpler constituents. These considerations suggest that the amination of a keto acid cannot be taken as proof of the function of the keto acid analogue as an intermediate in the normal biosynthesis of an amino acid.

The symmetric synthesis of racemic amino acids may not take place in the rat (184) and the unnatural isomers may even be inhibitory or toxic to animals (185), or microorganisms (167). However, the synthesis of the unnatural forms of leucine and valine by certain bacteria (186 to 189), and of *dl*-alanine (isolated as fumaryl-*dl*-alanine) by a mold (190) seems to be well established. The inactivity of the unnatural enantiomorphs of tryptophane and serine (171) for *Neurospora* suggests asymmetric synthesis of at least these amino acids, although symmetric synthesis followed by oxidative deamination is not excluded in other instances. The activity of unnatural isomers of amino acids for mutant strains of *Neurospora* is in general correlated

with the ease of their attack by *d*-amino oxidase from *Neurospora* (191).

Adaptation and Changes in Synthetic Capacity

The general concept of the genetic control of biosynthesis permits a satisfactory genetic interpretation of three of the four classes which Fries (107) suggested for fungi on the basis of their responses to growth factors, and which have in general been accepted (11, 58). Fungi with fully adequate synthetic capacities and those with absolute deficiencies may result from either full activity or complete loss or inactivity of genes controlling the syntheses. Those fungi with limited synthetic capacities which are therefore markedly stimulated by added growth factors may result from allelic forms of these genes with limited synthetic activity. The last group, those in which an apparently completely lacking synthetic capacity is restored under different environmental conditions, is less easily explained. [See also Schopfer (58), p. 198.] Some examples of the induction of such changes in synthetic capacity in fungi are the effects of salt concentration on thiamin synthesis by *Pythium butleri* (192), of temperature on Z-factor (hypoxanthine) synthesis by *Phycomyces* (193), and of the nitrogen source and acidity on biotin synthesis by *Mitrula paludosa* (16) and on the synthesis of biotin, inositol, and pyridoxin by *Trichophyton album* (194). As with yeast (195, 196) vitamins or amino acids may influence the synthesis of certain vitamins in fungi (12, 197).

Changes in which a synthetic ability is regained are not irreconcilable with the concept of genetic control of these syntheses. Certain syntheses may be likewise restored in *Neurospora* mutant strains by environmental changes apparently without affecting the genes concerned. This is seemingly true in the synthesis of pyridoxin by the "pyridoxinless" strain at a pH above 5.8 in the presence of ammonium salts (198) and in the synthesis of arginine by one of the "arginineless" mutants at pH of 6.7 or higher (169). In both cases the mutant genes are apparently unchanged.

Another phenomenon which possibly bears on this general problem is that of "adaptation." This term has been used to denote the ability of a mutant strain of *Neurospora* to redevelop a lost synthetic capacity after a definite lag period following inoculation (166). This phenomenon seems to be fairly common in a number of mutant strains of *Neurospora* (144, 166, 179), but in certain cases investigated the adapta-

tion apparently has not involved genetic changes, although others may be found to do so. This phenomenon of adaptation may be similar to "training" of yeasts or bacteria with resulting modifications in synthetic capacities (25, 79).

There are at least two possible explanations for such non-genetic changes in synthetic ability. One is based on the view that mutation of a gene to the recessive form may not result in a complete loss of function. In this case a modification of the environmental conditions might increase the rate or efficiency of the limiting synthesis (see also p. 690), similar to the influence of cultural conditions on the synthesis of riboflavin by yeast (75). The other possibility is that there may be more than one biochemical pathway by which a given compound can be synthesized. This has been suggested (166) as an explanation of the phenomenon of adaptation, but may equally well apply to synthetic abilities regained in response to environmental changes. In both cases latent or alternative mechanisms may become functional, possibly through the development of adaptive enzymes. The production of adaptive enzymes [see (199)] in genetically controlled yeast populations has recently been studied (200).

Inhibitions

Complex interrelationships between amino acids which result in inhibition of growth by certain amino acids and the antagonism of this effect by other amino acids are known in bacteria (126, 167, 168, 201 to 207). Analogous phenomena have been found in *Neurospora* (166). Inhibition of growth responses to vitamins by structurally related analogues has been noted in bacteria (143, 208 to 211) and in fungi (15, 20, 212). In general both types of inhibition seem to affect primarily the utilization of a vitamin or amino acid when the organism requires an outside source, although there are exceptions with amino acids (167, 168, 205, 206) and with sulfanilamide (20). In one type of inhibition, therefore, the effects are in some way related to the synthetic capacity of the organism, as clearly shown in the case of the pyridine analogue of thiamin, pyrithiamin. This inhibits most actively organisms with a requirement for intact thiamin, and has almost no effect on those with no requirement either for thiamin or for its components (212). The failure to inhibit the latter class of organisms seems not to be due to an increased synthesis of thiamin as contrasted with the effect of sulfanilamide on certain bacteria (106).

Cultural Specificity and Cultural Conditions Affecting Mold Products

There is no inherent reason why the genetically controlled reactions in *Neurospora* should be fundamentally different from analogous reactions in other fungi such as *Aspergillus* or *Penicillium,* in which genetic analysis is impossible as yet. Furthermore, it is probable that the biochemical processes involved in the formation of simple or complex organic acids, of pigments, and of antibiotic agents are qualitatively similar to those involved in the gene-controlled synthesis of vitamins and amino acids in *Neurospora.* The conclusion is therefore almost inescapable that the metabolism of other fungi must also be under more or less direct genetic control.

The available evidence indicates that the three perhaps too arbitrarily defined classes of mold products—acids, pigments, and certain antibiotic agents—which all arise ultimately from sugar, may have definite biochemical relationships (see p. 691). The biological production of these substances is apparently strictly analogous, in species and strain specificity, and in modification by environmental and cultural conditions, to the synthesis of vitamins by fungi.

The species, and even to a greater extent the strain, specificities of molds in the production of simple organic acids such as succinic, oxalic, and citric acids (1 to 5) have been repeatedly demonstrated (213 to 216). Similar specificities are shown in the production of more complex organic acids and pigments [see Raistrick (4)], and have more recently been found in the production of different antibiotic substances (217 to 224).

Recent investigations have also shown that a number of closely related acids, pigments, and antibiotic substances may be produced by several strains of the same species, or even by strains of different species, of fungi. This fact indicates that the syntheses of these substances is intimately correlated with metabolic reactions or sequences common perhaps to all species and strains. A number of such interrelationships in the production of complex organic acids and pigments have been described (4, 225 to 228). Strains of both *Aspergilli* and *Penicillia* may produce citrinin (213, 229, 230). Clavicin from *A. clavatus* (219, 231), claviformin from *P. claviforme* (232), and patulin from *P. patulum* (233) have been shown to be identical (234, 235). Antibiotic agents either closely related to or identical with penicillin, originally from *P. notatum* [for references see (223)] are apparently

produced also by *P. chrysogenum* (236) and by *A. flavus* (237, 238) and *A. flavipes* (239). In a number of instances it has been established, as has been pointed out (220, 240), that more than one antibiotic agent may be produced by a given strain (217, 223, 238), presumably by independent mechanisms.

The influence of cultural conditions in the production of organic acids and pigments by molds has been discussed in previous reviews (5, 241), and additional instances have been described (242 to 245). Similar examples of modification of the production of antibiotic substances by fungi are also known. The formation of aspergillic acid by *A. flavus* requires complex organic nitrogen (224), and the production of clavicin by *A. clavatus* is markedly affected by the pH of the medium (219). The production of flavicin (similar to or identical with penicillin) by *A. flavus* is influenced by the addition of corn steep water and by aeration (220). The production of penicillin is increased by the use of lactose as a carbon source (246), by certain nitrogen sources, by aeration, and by trace elements, especially zinc (223). Foster *et al.* (223) have shown that zinc catalyzes the complete oxidation of gluconic acid, as previously shown for fumaric acid (242), and so prevents the pH from falling to a level unfavorable for penicillin production, but favorable to the production of clavicin. Aeration has a similar effect on the production of aspergillic acid and penicillin (238).

Nature and Action of Antibiotic Agents from Fungi

An adequate understanding of the production and action of antibiotic substances must of necessity depend on the determination of their chemical structure. Although little information is at present available on certain products, especially penicillin, more is known of the chemical nature of others. It seems likely that most, if not all, will eventually be found to have definite relationships to previously known products of mold metabolism or to substances involved in this metabolism, so that both their origin and action will be understandable.

A number of previously known mold products, citrinin, penicillic acid, spinulosin, and fumigatin (247 to 250) have been found to have antibiotic action, and even more active synthetic analogues of fumigatin and spinulosin have been prepared (251). Penatin (240, 252, 253, 254) [notatin (255), coli-factor (256), penicillin B (257)] has been identified as a flavoprotein which owes its activity to the production of hydrogen peroxide (258, 259, 260). The antibacterial action of enzymatically produced hydrogen peroxide has been conclu-

sively demonstrated with xanthine oxidase (261, 262). Patulin (see above) has been identified as anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid, with the formula $C_7H_6O_4$ (233), and therefore related to kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone).

The structures of other antibiotic substances have not yet been established or revealed, although the empirical formulae of aspergillic acid, probably $C_{12}H_{20}N_2O_2$ (224), of gliotoxin (263), $C_{13}H_{14}N_2O_4S_2$ (264), and of helvolic acid, $C_{32}H_{44}O_8$ (265) are known. Two decomposition products of penicillin have been reported, penillic acid (266) and penicillamine hydrochloride, $C_6H_{11}O_4N.HCl$ (267). On the basis of this formula and from the chemical behavior of penicillamine, Abraham *et al.* (267) suggest that this portion of the penicillin molecule may be related to an amino sugar or to ascorbic acid. The relation of ascorbic and penicillic acids to the tetronic acids has been previously pointed out by Raistrick (4).

With the exception of clavicin (see above) the mode of action of these substances is unknown, although the available evidence suggests the inhibition of essential metabolic functions of susceptible organisms, as with the sulfa drugs, by competition with closely related substances involved in these functions. Raistrick (4) has pointed out the possible respiratory functions of a number of mold products, some of which have subsequently been shown to have antibiotic properties. The antagonistic relationships of iodinin (from *Chromobacterium iodinium,* 268) and of other *p*-diazine-di-N-oxides (269) to hydroxy-naphthoquinones and anthraquinones have been pointed out by McIlwain. Certain naphthoquinones which are related to vitamin K are essential growth factors for bacteria (270) and are synthesized by others (271), while a number of anthraquinone derivatives are produced by fungi (4). The bacteriostatic effect of sulfonamides has been correlated with their ability to form tautomeric quinone-like compounds (272). Tyrothricin may owe its activity to a quinone-like structure (273).

Studies on the specificity of action of a number of antibiotic agents (274, 275, 276) have supported these general relationships since certain agents (actinomycin, clavicin, and gliotoxin) act on certain fungi as well as on bacteria. The antagonism of these agents by pyridoxin or by peptone for *Ceratostomella ulmi* (276) seems likely to be indirect, through an increased synthesis of natural (and possibly metabolically essential) antagonists. Other reported relationships include the synergistic effects of *p*-aminobenzoic acid or sulfapyridine on peni-

cillin activity (277) and the inhibition of urease activity by purified penicillin (278). Although the relations of concentration of penicillin and streptothricin to activity are different, the two have an additive antibiotic effect (275) and both affect division of bacteria (275, 279).

In view of these various relationships it seems reasonable to conclude that the various mold products—acids, pigments, and antibiotic substances—may represent normal metabolic products or cell constituents, or perhaps represent closely related substances formed from these. The formation of a pigmented product from *p*-aminobenzoic acid by bacteria (280) may illustrate such a secondary reaction. The time relations in the production of patulin (clavicin) suggest that this antibiotic substance is excreted continually during growth (218). On the other hand, streptothricin, suggested to be a metabolic waste product (281) of *Actinomyces lavendulae,* seems rather to be a product of cell synthesis which is liberated by enzyme action or autolysis only after active growth has ceased (282). Similar time relations in the production of penicillin (223) suggest a possibly analogous situation. In any case it seems probable that the inherent capacities for the formation of these various substances are genetically controlled. As may also be true in the synthesis of vitamins and amino acids, the effects of environmental conditions can be tentatively attributed in certain cases to the modification of metabolic reactions, resulting in the presence of varying amounts of intermediates required for the syntheses. The effects of these intermediates would be analogous to the effects of pimelic acid and cystine on biotin synthesis (137), of complex nitrogenous compounds on aspergillic acid production (224), of certain amino acids on gramicidin and tyrocidine synthesis by bacteria (283), and on streptothricin production by actinomycetes (281, 282). Another possibility is that the rates of specific syntheses may be increased under definite metabolic conditions modifiable by aeration, pH, or other conditions, as indicated for the synthesis of certain vitamins. A third possibility is that certain cultural conditions may facilitate selection of certain advantageous nuclear combinations from a complex heterocaryon (see p. 675). Foster *et al.* (223) have shown that the "degeneration" of cultures of *P. notatum* (284) involves the production of sub-strains which vary widely in the production of spores, pigments, and penicillin. Similar "variant" strains of *A. flavus* have been derived which vary in the production of aspergillic acid (285). The occurrence of this phenomenon in genetically homogeneous strains would necessitate an extremely high frequency of gene mutation, and it seems more

likely that each strain is a heterocaryon and that selection results in redistribution of heterogeneous nuclei so that the resulting sub-strains more nearly approach nuclear homogeneity.

Interrelationships of Mold Products

The wide variety of acids and pigments formed by various species and strains of fungi has provided a rich field for chemical investigations of the nature of these products. It is felt that a tentative interpretation of the biogenetic relationships of these mold products is now possible. Although such an attempt must necessarily be speculative and incomplete, it may prove of value as a working hypothesis.

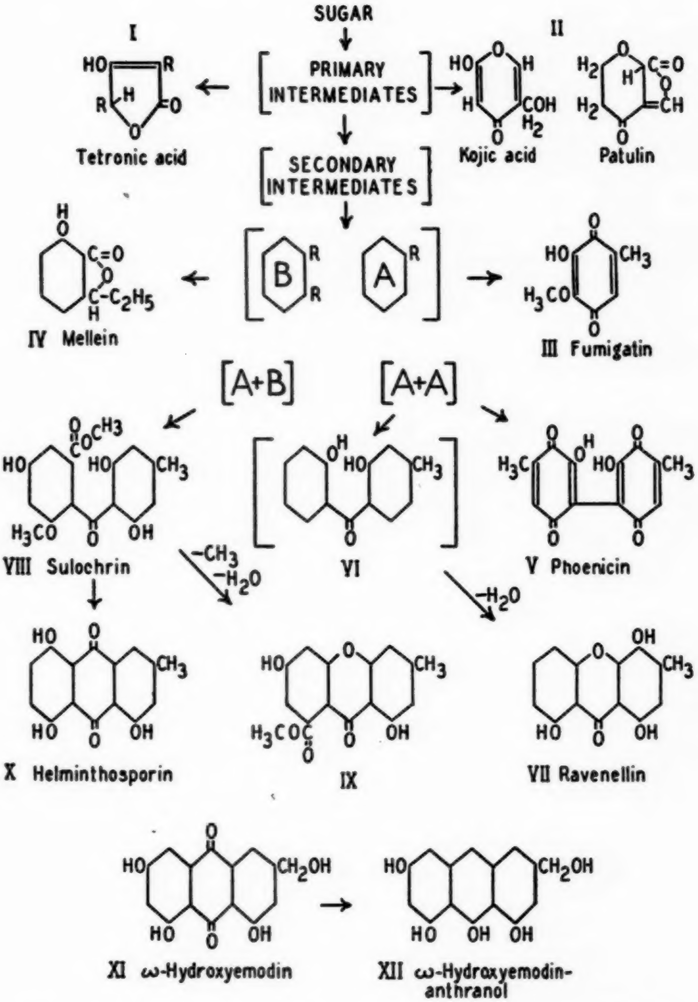
All the products obtained from molds must ultimately have come from sugar, and the relatively high yields of certain compounds such as kojic acid, catenarin, and helminthosporin suggest, as pointed out by Raistrick (4), that the compounds[3] represent end products of carbohydrate metabolism. Most of the mold products so far reported may be classified as derivatives of a rather limited number of chemical groups. Raistrick has pointed out the occurrence of analogously substituted rings in a number of substances belonging to different groups. This suggests that the various ring systems are built from common structural units and that the syntheses involve analogous biochemical reactions.

The strain specificities of fungi in the production of the various compounds, and the indicated relationships between these substances, together with the general theoretical relationships between biosynthesis and genes, suggest that the synthesis of these mold products is genetically controlled. The genetic control of biosyntheses, many of which also involve the formation of specifically or characteristically substituted aromatic rings, has been demonstrated in the mold *Neurospora*. Syntheses in plants of heterocyclic aromatic rings (anthocyanins, anthoxanthins, and chalcones) with different substituent groups have been definitely shown to be under the direct control of specific genes governing different biochemical steps (61, 286). All three types of these plant pigments are thought to come from common precursors which are formed more or less directly from sugars. The occurrence of the differently modified pigments in different plants is

[3] Specific references will be given here only for compounds not included in Raistrick's review (4). Page numbers in parentheses after a compound or type of compound refer to pages in Raistrick's review.

analogous to that of various mold products in different strains and species of molds.

The proposed interrelationships of mold products would also involve the gene-controlled, perhaps sequential, formation of a number of intermediate aliphatic compounds from sugars or sugar derivatives. The various end products could arise from these intermediate compounds through different types of ring closures. Minor alterations in structure, such as effected by oxidation and methylation, as in the biosynthesis of plant pigments, might occur either before or after ring closure, but would likewise be controlled by specific genes. The accompanying diagram presents some of the possible interrelationships between the different chemical groups, as well as a few examples of relationships and modifications within the groups. There are two main groups of relatively simple alicyclic compounds which could be formed fairly directly from hexose sugars or their metabolic products: the γ-lactone tetronic acid derivatives (I) (p. 583), possibly including terrein, minioluteic acid (287), and spiculisporic acid (288); and kojic acid and patulin (II) (233). Most of the other known products are derivable from aromatic intermediates of two general types, represented as A and B, which may be formed by ring closures in different positions. These and other bracketed compounds in the diagram represent hypothetical type intermediates. Monocyclic products of type A include a number of hydroxybenzoic and hydroxycinnamic acid derivatives (p. 585), and the toluquinones, fumigatin (III), and spinulosin (p. 572). Monocyclic products of type B include 6-hydroxy-2-methylbenzoic acid, mellein (IV) (p. 585), and 3,5-dihydroxyphthalic acid (289). The more complicated ring structures could be formed by condensations of two intermediates, either A + A or A + B. Only one or two representatives of each group are included in the diagram and discussion, since the other known representatives differ from the type compound primarily in the number or location of hydroxy or methoxy groups. One type of condensation of intermediates A + A, perhaps those also common to the synthesis of the toluquinones, would result in the formation of phoenicin (V) (p. 573). Another type of condensation of intermediates A + A, possibly by way of a benzophenone such as VI, would lead to the formation of hydroxyxanthones (p. 580), ravenellin (VII), and rubrofusarin, in which the linkage is structurally analogous to that in plant anthoxanthins. The formation of VII from VI is similar to the production *in vivo* from sulochrin (VIII) of the hydroxyxanthone (IX) (p. 589). Benzophenone derivatives



which could be formed by condensations of A + B include sulochrin (VIII) and the related chlorine compounds geodin and erdin (p. 587). The linkage in this class is analogous to that in plant chalcones. A sec-

ond similar ring closure in benzophenone type intermediates similar to VIII would result in the formation of 2-methyl anthraquinones (p. 578), such as helminthosporin (X) and ω-hydroxyemodin (XI), or their reduction products, the anthranols (p. 579), such as penicilliopsin (290), which is probably a dimeride of ω-hydroxyemodin anthranol (XII).

If these general relationships hold it would then be possible to account for the amounts of the various mold products in certain cases and for their diversity and strain specificity on the basis of gene-controlled biochemical reactions. This hypothesis would be supported by evidence that the simpler compounds are actually precursors of or related to precursors of the more complex substances, by competition for common precursors as with plant pigments, or most satisfactorily, by the demonstration of the genetic control of these specific reactions in fungi.

Miscellaneous

No additional information regarding the mechanism of synthesis of succinic and citric acids by fungi has been reported since the demonstration by Foster *et al.* (291) that carbon dioxide is involved. This work and its implications have been reviewed by Krebs (292). The participation of four carbon dicarboxylic acids in respiration and growth of *Neurospora* has been demonstrated (96). The results were similar to those obtained with *Avena* (293). The rapid uptake of inorganic phosphate by *A. niger* during the aerobic production of citric and gluconic acids seems to involve the formation of organic phosphates or of polyphosphates (294). Further studies on alcohol production and on dehydrogenations by *Fusaria* have been reported (295, 296, 297). The results are not inconsistent with the operation *in vivo* of a mechanism similar to that in cell-free yeast preparations, including the function of diphosphopyridine nucleotide (298). As in yeast, sulfur acts as a hydrogen acceptor (299). The enzymatic breakdown of alanine by *Fusaria* is also apparently normal (300).

Mold metabolic products isolated include trimethylsulochrin (301), and caldariomycin, $C_5H_8O_2Cl_2$ (302). The structures of the anthraquinones, erythroglaucin and catenarin, have been established (303) and the structure of stipitatic acid has been investigated (304, 305). A number of lipid and polysaccharide constituents of mold mycelium have been reported. These include a crystalline fungus-cerebrin from *A. sydowi* (306), further investigations of the lipids of *Blastomyces*

dermatiditis (307), the isolation from *Penicillia* of the polysaccharides luteose (308) and sclerotiose (309), and the isolation of an immunologically active polysaccharide from *Coccidioides immitis* (310).

The biochemical adaptability of fungi is illustrated by a mold which will grow in saturated copper sulfate or 2.5 *N* sulfuric acid solutions (311), and by the first reported instance of the production by a fungus of methyl mercaptan from sulfate (312).

Different methods of assays for penicillin have been compared (313), and modifications of the Oxford cup method (314) have been suggested for penicillin (315) and for streptothricin (316). Assays for antiobiotic activity by turbidimetric methods (317, 318) and indirect methods which depend on inhibition of bacterial luminescence (285, 319) or hemolysin production (320, 321) have been suggested. The inactivation of penicillin for sterility tests (322) has been further investigated (323, 324). Methods for the production of citrinin (247, 325) and penicillin (326), and for the clinical use of crude penicillin (327) and of penicillin esters (328, 329) have been developed.

Microbiological assays for certain amino acids and vitamins have been developed in which either the rate of growth (330) or the dry weight of mycelium of mutant strains of *Neurospora* is measured. These include assays for leucine (179, 331), *p*-aminobenzoic acid (332), pyridoxin (131, 333), and choline (22).

LITERATURE CITED

1. Iwanoff, N. N., *Ann. Rev. Biochem.*, **1**, 675–97 (1932)
2. Iwanoff, N. N., and Zwetkoff, E. S., *Ann. Rev. Biochem.*, **2**, 521–40 (1933)
3. Iwanoff, N. N., and Zwetkoff, E. S., *Ann. Rev. Biochem.*, **5**, 585–612 (1936)
4. Raistrick, H., *Ann. Rev. Biochem.*, **9**, 571–92 (1940)
5. Birkinshaw, J. H., *Biol. Revs. Cambridge Phil. Soc.*, **12**, 357–92 (1937)
6. Beadle, G. W., and Tatum, E. L., *Proc. Natl. Acad. Sci. U.S.*, **27**, 499–506 (1941)
7. Williams, R. J., *Science*, **93**, 412–14 (1941)
8. Peterson, W. H., *Biol. Symposia*, **5**, 31–43 (1941)
9. van Niel, C. B., *Ann. Rev. Biochem.*, **12**, 551–86 (1943)
10. Robbins, W. J., and Kavanagh, V., *Ann. Rev. Biochem.*, **10**, 491–508 (1941)
11. Robbins, W. J., and Kavanagh, V., *Botan. Rev.*, **8**, 411–71 (1942)
12. Robbins, W. J., and Ma, R., *Arch. Biochem.*, **1**, 219–29 (1942–43)
13. Robbins, W. J., and Ma, R., *Bull. Torrey Botan. Club*, **70**, 190–97 (1943)
14. Burkholder, P. R., and McVeigh, I., *Science*, **95**, 127–28 (1942)

15. Robbins, W. J., and Ma, R., *Bull. Torrey Botan. Club,* **69**, 342–52 (1942)
16. Fries, N., *Nature,* **152**, 105–6 (1943)
17. Burkholder, P. R., and Moyer, D., *Bull. Torrey Botan. Club,* **70**, 372–77 (1943)
18. Robbins, W. J., Mackinnon, J. E., and Ma, R., *Bull. Torrey Botan. Club,* **69**, 509–21 (1942)
19. Williams, R. J., *Ann. Rev. Biochem.,* **12**, 305–52 (1943)
20. Tatum, E. L., and Beadle, G. W., *Proc. Natl. Acad. Sci. U.S.,* **28**, 234–43 (1942)
21. Tatum, E. L., and Beadle, G. W., *Growth,* **6**, 27–35 (1942)
22. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.,* **150**, 325–33 (1943)
23. Burkholder, P. R., *Am. J. Botany,* **30**, 206–21 (1943)
24. Leonian, L. H., and Lilly, V. G., *Am. J. Botany,* **29**, 459–64 (1942)
25. Leonian, L. H., and Lilly, V. G., *J. Bact.,* **45**, 329–39 (1943)
26. Burkholder, P. R., *Proc. Natl. Acad. Sci. U.S.,* **29**, 166–72 (1943)
27. Rogosa, M., *J. Bact.,* **45**, 459–60 (1943)
28. Lavollay, J., and Laborey, F., *Ann. fermentations,* **6**, 129–42 (1941)
29. Kitavin, G. S., *Compt. rend. acad. sci. U.R.S.S.,* **28**, 517–18 (1940)
30. Carpenter, C. C., and Friedlander, E. W., *Science,* **95**, 625 (1942)
31. Tatum, E. L. (Unpublished data)
32. Lewis, R. W., *Papers Mich. Acad. Sci.,* **24**, Pt. 1, 31–35 (1938)
33. Gupta, G. C. D., and Guha, B. C., *Ann. Biochem. Exptl. Med.,* **1**, 14–26 (1941)
34. Roberts, J. S., and Roberts, E., *Soil Sci.,* **48**, 135–39 (1939)
35. Kerl, I., *Z. Botan.,* **31**, 129–74 (1937)
36. Thimann, K. V., and Dolk, H., *Biol. Zentr.,* **53**, 49–66 (1933)
37. Bunning, E., *Ber. deut. botan. Ges.,* **52**, 423–44 (1934)
38. Robbins, W. J., *Botan. Gaz.,* **102**, 520–35 (1941)
39. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.,* **28**, 4–8 (1942)
40. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.,* **28**, 65–69 (1942)
41. Robbins, W. J., *Proc. Natl. Acad. Sci. U.S.,* **29**, 201–2 (1943)
42. Melin, E., and Norkrans, B., *Svensk Botan. Tid.,* **36**, 271–86 (1942)
43. Schopfer, W. H., *Compte rend., soc. phys. hist. natl. Genève,* **59**, 101 (1942)
44. Schopfer, W. H., *Plants and Vitamins,* p. 271 (Chronica Botanica, Waltham, Mass., 1943)
45. Tytell, A. A., and Gould, B. S., *J. Bact.,* **42**, 513–26 (1940)
46. Franke, W., and Deffner, M., *Ann.,* **541**, 117–50 (1939)
47. Goddard, D. R., and Smith, P. E., *Plant Physiol.,* **13**, 241–64 (1938)
48. Robbins, W. J., Kavanagh, V. W., and Kavanagh, F., *Botan. Gaz.,* **104**, 224–42 (1942)
49. Giese, A. C., and Tatum, E. L., *Collecting Net,* **17**, 86–88 (1942)
50. Hills, G. M., *Biochem. J.,* **37**, 418–24 (1943)
51. Hellinga, J. J. A., *Proc. Acad. Sci. Amsterdam,* **43**, 249–66, 267–76 (1940)
52. Allen, P. J., and Goddard, D. R., *Science,* **88**, 192–93 (1938)
53. Allen, P. J., and Goddard, D. R., *Am. J. Botany,* **25**, 613–21 (1938)
54. Allen, P. J., *Am. J. Botany,* **29**, 425–35 (1942)

55. Lwoff, A., *Ann. inst. Pasteur,* **61**, 580-617 (1938)
56. Knight, B. C. J. G., "Bacterial Nutrition," *Med. Research Council (Brit.), Special Rept. Series,* No. **210**, 182 pp. (1936)
57. Schopfer, W. H., *Ergeb. Biol.,* **16**, 1-172 (1939)
58. Schopfer, W. H., *Plants and Vitamins,* 293 pp. (Chronica Botanica, Waltham, Mass., 1943)
59. Woods, A. M., Taylor, J., Hofer, M. J., Johnson, G. A., Lane, R. L., and McMahan, J. R., *Univ. Texas Bull.,* No. **4337**, 84-86 (1942)
60. Beadle, G. W., and Tatum, E. L., *Am. Naturalist,* **75**, 107-16 (1941)
61. Lawrence, W. J. C., and Price, J. R., *Biol. Revs. Cambridge Phil. Soc.,* **15**, 35-58 (1940)
62. Wright, S., *Physiol. Revs.,* **21**, 487-527 (1941)
63. Ephrussi, B., *Quart. Rev. Biol.,* **17**, 327-38 (1942)
64. Lindegren, C. C., *Iowa State Coll. J. Sci.,* **16**, 271-90 (1942)
65. Dodge, B. O., *Science,* **90**, 379-85 (1939)
66. Tatum, E. L., *Stanford Med. Bull.,* **2**, 1-4 (1944)
67. Winge, O., and Laustsen, O., *Compt. rend. trav. lab. Carlsberg., Ser. physiol.,* **22**, 337-52 (1939)
68. Deere, C. J., *J. Bact.,* **37**, 473-88 (1939)
69. Atwood, S. A., and Sullivan, J. T., *J. Heredity,* **34**, 311-20 (1943)
70. Sawin, P. B., and Glick, D., *Proc. Natl. Acad. Sci. U.S.,* **29**, 55-59 (1943)
71. Lindegren, C. C., and Lindegren, G., *J. Bact.,* **46**, 405-20 (1943)
72. Ragosa, M., *J. Bact.,* **46**, 435-40 (1943)
73. Wolfand, F. T., and Shoup, C. S., *Mycologia,* **35**, 192-200 (1943)
74. Stier, T. J. B., and Castor, J. G. B., *J. Gen. Physiol.,* **25**, 229-33 (1941)
75. Burkholder, P. R., *Arch. Biochem.,* **3**, 121-29 (1943)
76. Robbins, W. J., and Ma, R., *Bull. Torrey Botan. Club,* **68**, 446-62 (1941)
77. Barker, H. A., *Ann. Rev. Biochem.,* **10**, 553-86 (1941)
78. Giese, A. C., *J. Bact.,* **46**, 323-31 (1943)
79. Koser, S. A., and Wright, M. H., *J. Bact.,* **46**, 239-49 (1943)
80. Luria, S. E., and Delbruck, M., *Genetics,* **28**, 491-511 (1943)
81. Various Authors, "Genetics of Pathogenic Organisms," *Pub. Am. Assoc. Adv. Sci.,* No. **12** (Science Press, 1940)
82. Stakman, E. C., Kernkamp, M. F., King, T. H., and Martin, W. J., *Am. J. Botany,* **30**, 37-48 (1943)
83. Wellman, F. L., and Blaisdell, D. J., *U.S. Dept. Agr. Tech. Bull.,* No. **705** (1940)
84. Wellman, F. L., and Blaisdell, D. J., *Phytopathology,* **31**, 103-20 (1941)
85. Wellman, F. L., *Phytopathology,* **33**, 1004-17 (1943)
86. Gottlieb, D., and Hart, H., *Phytopathology,* **33**, 724-28 (1943)
87. Macrae, R., *Can. J. Research,* **20**, Sec. C, 411-34 (1942)
88. Foster, J. W., and Waksman, S. A., *J. Am. Chem. Soc.,* **61**, 127-35 (1939)
89. Benedek, T., *Mycologia,* **35**, 222-42 (1943)
90. Thren, R., *Arch. mikrobiol.,* **12**, 192-228 (1941)
91. Nickerson, W. J., and Thimann, K. V., *Am. J. Botany,* **28**, 617-21 (1941)
92. Lindegren, C. C., and Lindegren, G., *Ann. Missouri Botan. Garden,* **30**, 453-68 (1943)
93. Nickerson, W. J., and Thimann, K. V., *Am. J. Botany,* **30**, 94-101 (1943)

94. English, J., Jr., Bonner, J., and Haagen-Smit, A. J., *Science*, **90**, 329 (1939)
95. Haagen-Smit, A. J., and Thimann, K. V., *J. Cellular Comp. Physiol.*, **11**, 389–407 (1938)
96. Ryan, F. J., Tatum, E. L., and Giese, A. C., *J. Cellular Comp. Physiol.*, **23**, 83–94 (1944)
97. Raper, A., *Am. J. Botany*, **29**, 159–66 (1942)
98. Raper, J. R., and Haagen-Smit, A. J., *J. Biol. Chem.*, **143**, 311–20 (1942)
99. Kuhn, R., and Moewus, F., *Ber. deut. chem. Ges.*, **73**, 547–59 (1939)
100. Emerson, R., and Fox, D. L., *Proc. Roy. Soc. (London), B*, **128**, 275–93 (1940)
101. Nakazawa, R., and Simo, M., *J. Agr. Chem. Soc. Japan*, 14, 895–910 (1938)
102. Steinberg, R. A., and Thom, C., *J. Heredity*, **31**, 61–63 (1940)
103. Steinberg, R. A., and Thom, C., *J. Agr. Research*, **64**, 645–52 (1942)
104. Bauch, R., *Naturwissenschaften*, **29**, 687–88 (1941)
105. Levan, A., and Sandwall, C. G., *Hereditas*, **29**, 164–78 (1943)
106. Landy, M., Larkum, N., Oswald, E. J., and Streightoff, F., *Science*, **97**, 265–67 (1943)
107. Fries, N., *Symbolae Botan. Upsalienses*, 3, No. 1, 188 pp. (1938)
108. Kögl, F., and Fries, N., *Z. physiol. Chem.*, **249**, 93–110 (1937)
109. Müller, W. F., and Schopfer, W. H., *Compt. rend. acad. sci. Paris*, **205**, 687 (1937)
110. Müller, F. W., *Ber. schweiz. botan. Ges.*, **51**, 165–256 (1941)
111. Utiger, H., and Schopfer, W. H., *Compt. rend. soc. phys. hist. nat. Genève*, **58**, 284–88 (1941)
112. Hansen, H. N., and Smith, R. E., *Phytopathology*, **22**, 953–64 (1932)
113. Hansen, H. N., *Mycologia*, **30**, 442–55 (1938)
114. Powers, E. L., Jr., *Am. Midland Naturalist*, **30**, 175–95 (1943)
115. Dodge, B. O., *Bull. Torrey Botan. Club*, **69**, 75–91 (1942)
116. Beadle, G. W., and Coonradt, V. L., *Genetics*, **29**, 291–308 (1944)
117. Schoenheimer, R., "The Dynamic State of Body Constituents," *Harvard Univ. Monograph Med. Pub. Health* (Cambridge, 1942)
118. Barnes, F. W., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **151**, 123–39 (1943)
119. Bonner, J., and Buchman, E. R., *Proc. Natl. Acad. Sci. U.S.*, **25**, 164–71 (1939)
120. Robbins, W. J., and Kavanagh, F., *Proc. Natl. Acad. Sci. U.S.*, **27**, 423–27 (1941)
121. Robbins, W. J., *Proc. Natl. Acad. Sci. U.S.*, 27, 419–22 (1941)
122. Kavanagh, F., *Bull. Torrey Botan. Club*, **69**, 669–91 (1942)
123. Bonner, J., and Buchman, E. R., *Proc. Natl. Acad. Sci. U.S.*, **24**, 431–38 (1938)
124. Robbins, W. J., *Bull. Torrey Botan. Club*, **65**, 267–76 (1938)
125. Kidder, G. W., and Dewey, V. C., *Growth*, **6**, 405–18 (1942)
126. Snell, E. E., and Guirard, B. M., *Proc. Natl. Acad. Sci. U.S.*, **29**, 66–73 (1943)
127. Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519–30 (1942)

128. Snell, E. E., *Proc. Soc. Exptl. Biol. Med.*, **51**, 356–58 (1942)
129. Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc. Exptl. Biol. Med.*, **54**, 123–25 (1943)
130. Robbins, W. J., and Ma, R., *Proc. Natl. Acad. Sci. U.S.*, **29**, 172–76 (1943)
131. Stokes, T. J., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, **150**, 17–24 (1943)
132. Wieland, T., and Moller, E. F., *Z. physiol. Chem.*, **269**, 227–35 (1941)
133. Wieland, T., and Moller, E. F., *Z. physiol. Chem.*, **272**, 233–38 (1942)
134. McIlwain, H., *Biochem. J.*, **37**, XIII (1943)
135. Ivanovics, G., *Z. physiol. Chem.*, **276**, 33–35 (1942)
136. du Vigneaud, R., Dittmer, K., Hague, E., and Long, B., *Science*, **96**, 186–87 (1942)
137. Eakin, R. E., and Eakin, E. A., *Science*, **96**, 187–88 (1942)
138. Wright, L. D., *Proc. Soc. Exptl. Biol. Med.*, **51**, 27 (1942)
139. Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exptl. Biol. Med.*, **51**, 204–5 (1942)
140. Robbins, W. J., and Ma, R., *Science*, **96**, 406–7 (1942)
141. Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, **99**, 203–5 (1944)
142. Lilly, V. G., and Leonian, L. H., *Science*, **99**, 205–6 (1944)
143. McIlwain, H., *Nature*, **151**, 270–72 (1943)
144. Wyss, O., Lilly, V. G., and Leonian, L. H., *Science*, **99**, 18–19 (1944)
145. Blinks, L. R., *Ann. Rev. Physiol.*, **4**, 1–24 (1942)
146. Wyss, O., Rubin, M., and Strandskov, F. B., *Proc. Soc. Exptl. Biol. Med.*, **52**, 155–58 (1943)
147. Borsook, H., and Dubnoff, J. W., *Ann. Rev. Biochem.*, **12**, 183–204 (1943)
148. Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153**, 61–69 (1944)
149. Richardson, G. M., *Biochem. J.*, **30**, 2184–90 (1936)
150. Stokstad, E. L. R., *J. Biol. Chem.*, **139**, 475–76 (1941)
151. Woolley, D. W., and Peterson, W. H., *J. Biol. Chem.*, **121**, 507–20 (1937)
152. Woolley, D. W., and Peterson, W. H., *J. Biol. Chem.*, **118**, 363–70 (1937)
153. Bohonos, N., Woolley, D. W., and Peterson, W. H., *Arch. Biochem.*, **1**, 319–24 (1942)
154. Skinner, C. E., and Muller, A. E., *J. Nutrition*, **19**, 333–44 (1940)
155. Burkholder, P. R., and McVeigh, I., *Am. J. Botany*, **27**, 634–40 (1940)
156. Fildes, P., and Richardson, G. M., *Brit. J. Exptl. Path.*, **16**, 326–35 (1935)
157. Mueller, J. H., and Kapnick, I., *J. Bact.*, **30**, 525–34 (1935)
158. Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, **98**, 266 (1943)
159. Shankman, S., Dunn, M. S., and Rubin, L. B., *J. Biol. Chem.*, **151**, 511–14 (1943)
160. Lankford, C. E., and Snell, E. E., *J. Bact.*, **45**, 410–11 (1943)
161. Rose, W. C., Haines, W. J., Johnson, E., and Warner, D. T., *J. Biol. Chem.*, **148**, 457–58 (1943)
162. Bonner, D., Houlahan, M. B., and Sprague, G. F. (Unpublished data)

163. Steinberg, R. A., *J. Agr. Research*, **64**, 455–75 (1942)
164. Steinberg, R. A., *J. Agr. Research*, **64**, 615–33 (1942)
165. Raaf, H., *Arch. Mikrobiol.*, **12**, 131–82 (1941)
166. Bonner, D., Tatum, E. L., and Beadle, G. W., *Arch. Biochem.*, **3**, 71–91 (1943)
167. Gladstone, G. P., *Brit. J. Exptl. Path.*, **20**, 189–200 (1939)
168. Doudoroff, M., *Proc. Soc. Exptl. Biol. Med.*, **53**, 73–75 (1943)
169. Srb, A., and Horowitz, N. H., *J. Biol. Chem.* (In press)
170. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33–66 (1932)
171. Tatum, E. L., and Bonner, D., *Proc. Natl. Acad. Sci. U.S.*, **30**, 30–37 (1944)
172. Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, **151**, 273–80 (1943)
173. Krebs, H. A., Hafez, M. M., and Eggleston, L. V., *Biochem. J.*, **36**, 306–10 (1942)
174. Binkley, F., and du Vigneaud, V., *J. Biol. Chem.*, **144**, 507–11 (1942)
175. Stetten, D., *J. Biol. Chem.*, **144**, 501–6 (1942)
176. Tatum, E. L., Bonner, D., and Beadle, G. W., *Arch. Biochem.*, **3**, 477–78 (1944)
177. Fildes, P., *Brit. J. Exptl. Path.*, **22**, 293–98 (1941)
178. Snell, E. E., *Arch. Biochem.*, **2**, 389–94 (1943)
179. Regnery, D., *J. Biol. Chem.* (In press)
180. Lipmann, F., and Perlman, G. E., *Arch. Biochem.*, **1**, 41–50 (1942)
181. Woods, D. D., *Biochem. J.*, **29**, 640–48, 649–55 (1935)
182. Moss, A. R., *J. Biol. Chem.*, **137**, 739–44 (1941)
183. Stephenson, M., *Bacterial Metabolism*, 2nd Ed., p. 162 (Longmans, Green & Co., London, 1939)
184. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **151**, 507–10 (1943)
185. Albanese, A. A., and Irby, V., *Science*, **98**, 286–88 (1943)
186. Lipmann, F., Hotchkiss, R. D., and Dubos, R. J., *J. Biol. Chem.*, **141**, 163–69 (1941)
187. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 313–17 (1943)
188. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 86–91 (1943)
189. Christensen, H. N., *J. Biol. Chem.*, **151**, 319–24 (1944)
190. Birkinshaw, J. H., Raistrick, H., and Smith, G., *Biochem. J.*, **36**, 829–35 (1942)
191. Horowitz, N. H., *J. Biol. Chem.* (In press)
192. Robbins, W. J., and Kavanagh, V., *Bull. Torrey Botan. Club*, **65**, 453–61 (1938)
193. Robbins, W. B., *Botan. Gaz.*, **101**, 428–49 (1939)
194. Schopfer, W. H., and Blumer, S., *Compt. rend. soc. phys. hist. nat. Genève*, **59**, 106 (1942)
195. Williams, R. J., *Biol. Revs. Cambridge Phil. Soc.*, **16**, 49–80 (1941)
196. Koser, L. A., and Wright, M. H., *Proc. Soc. Exptl. Biol. Med.*, **53**, 249–51 (1943)
197. Mosher, W. A., Saunders, D. H., Kingery, L. B., and Williams, R. J., *Plant Physiol.*, **11**, 795–806 (1936)

198. Stokes, J. L., Foster, J. W., and Woodward, C. R., Jr., *Arch. Biochem.*, **2**, 235–45 (1943)
199. Dubos, R., *Bact. Rev.*, **4**, 1–16 (1940)
200. Spiegelman, S., Lindegren, C. C., and Hedgecock, L., *Proc. Natl. Acad. Sci. U.S.*, **30**, 13–23 (1944)
201. Feeney, R. E., and Strong, F. M., *J. Am. Chem. Soc.*, **64**, 881–84 (1942)
202. Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **143**, 655–62 (1942)
203. Burrows, W., *J. Infectious Diseases*, **70**, 126–30 (1942)
204. Lorr, J. A., and Burrows, W., *J. Infectious Diseases*, **71**, 89–96 (1942)
205. Doudoroff, M., *J. Bact.*, **44**, 451–59 (1942)
206. Pelczar, M. J., Jr., and Porter, J. R., *Arch. Biochem.*, **2**, 323–32 (1943)
207. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exptl. Biol. Med.*, **52**, 36–38 (1943)
208. Snell, E. E., *J. Biol. Chem.*, **141**, 121–28 (1941)
209. Nielsen, N., and Johansen, G., *Naturwissenschaften*, **31**, 325 (1943); *Chem. Abstracts*, **38**, 138 (1944)
210. Pollack, M. A., *J. Am. Chem. Soc.*, **65**, 1335–39 (1943)
211. Wyss, O., *J. Bact.*, **46**, 483–84 (1943)
212. Woolley, D. W., and White, A. G. C., *J. Exptl. Med.*, **78**, 489–97 (1943)
213. Raistrick, H., and Smith, G., *Biochem. J.*, **29**, 606–11 (1935)
214. Calam, C. T., Oxford, A. E., and Raistrick, H., *Biochem. J.*, **33**, 1488–95 (1939)
215. Knobloch, H., *Biochem. Z.*, **307**, 278–84 (1941)
216. Knobloch, H., and Mayer, H., *Biochem. Z.*, **307**, 285–92 (1941)
217. Waksman, S. A., and Horning, E. S., *Mycologia*, **35**, 47–65 (1943)
218. Waksman, S. A., and Schatz, A., *Proc. Natl. Acad. Sci. U.S.*, **29**, 74–79 (1943)
219. Waksman, S. A., Horning, E. S., and Spencer, E. L., *J. Bact.*, **45**, 233–48 (1943)
220. Waksman, S. A., and Bugie, E., *Proc. Natl. Acad. Sci. U.S.*, **29**, 282–88 (1943)
221. Wilkins, W. H., and Harris, G. C. M., *Brit. J. Exptl. Path.*, **23**, 166–69 (1942)
222. Wilkins, W. H., and Harris, G. C. M., *Brit. J. Exptl. Path.*, **24**, 141–42 (1943)
223. Foster, J. W., Woodruff, H. B., and McDaniel, L. E., *J. Bact.*, **46**, 421–34 (1943)
224. White, E. C., and Hill, J. H., *J. Bact.*, **45**, 433–44 (1943)
225. Anslow, W. K., and Raistrick, H., *Biochem. J.*, **32**, 2288–89 (1938)
226. Curtin, T., Fitzgerald, G., and Reilly, J., *Biochem. J.*, **34**, 1605–7 (1940)
227. Curtin, T., Fitzgerald, G., and Reilly, J., *Biochem. J.*, **34**, 1607–10 (1940)
228. Quilico, A. Q., and Panizzi, L., *Ber. deut. chem. Ges.*, **76**, 348–57 (1943)
229. Hetherington, A. C., and Raistrick, H., *Phil. Trans. Roy. Soc. London*, B., **220**, 269–95 (1931)
230. Timonin, M. I., *Science*, **96**, 494 (1942)
231. Waksman, S. A., Horning, E. S., and Spencer, E. L., *Science*, **96**, 202–3 (1942)

232. Chain, E., Florey, H. W., and Jennings, M. A., *Brit. J. Exptl. Path.*, **23**, 202–5 (1942)
233. Birkinshaw, J. H., Bracken, A., Michael, S. E., and Raistrick, H., *Lancet*, **2**, 625–30 (1943)
234. Bergel, F., Morrison, A. L., Moss, A. R., Klein, R., Rinderknecht, H., and Ward, J. L., *Nature*, **152**, 750 (1943)
235. Hooper, I. R., Anderson, H. W., Skell, P., and Carter, H. E., *Science*, **99**, 16 (1944)
236. Smith, L. D., *J. Franklin Inst.*, **234**, 396–402 (1942)
237. Bush, M. T., and Goth, A. G., *J. Pharmacol.*, **78**, 164–69 (1943)
238. McKee, C. M., and MacPhillamy, H. P., *Proc. Soc. Exptl. Biol. Med.*, **53**, 247–48 (1943)
239. White, E. C., *Proc. Soc. Exptl. Biol. Med.*, **54**, 258–59 (1943)
240. Kocholaty, W., *Arch. Biochem.*, **2**, 73–86 (1943)
241. Foster, J. W., *Botan. Rev.*, **5**, 203–39 (1939)
242. Foster, J. W., and Waksman, S. A., *J. Bact.*, **37**, 599–617 (1939)
243. Bernhauer, K., Iglauer, A., Knobloch, H., and Zippelius, O., *Biochem. Z.*, **303**, 300–7 (1940)
244. Bernhauer, K., Iglauer, A., and Knobloch, H., *Biochem. Z.*, **307**, 298–306 (1941)
245. Reilley, D., and Curtin, T. P., *Biochem. J.*, **37**, 36–39 (1943)
246. Taylor, H. G., *Proc. Soc. Exptl. Biol. Med.*, **52**, 299–301 (1943)
247. Raistrick, H., and Smith, G., *Chemistry & Industry*, **60**, 828–30 (1941)
248. Oxford, A. E., Raistrick, H., and Smith, G., *Chemistry & Industry*, **61**, 22–24 (1942)
249. Oxford, A. E., *Chemistry & Industry*, **61**, 48–51 (1942)
250. Oxford, A. E., *Chemistry & Industry*, **61**, 128–29 (1942)
251. Oxford, A. E., *Chemistry & Industry*, **61**, 189–92 (1942)
252. Kocholaty, W., *J. Bact.*, **44**, 469–77 (1942)
253. Kocholaty, W., *J. Bact.*, **44**, 143 (1942)
254. Kocholaty, W., *Science*, **97**, 186–87 (1943)
255. Coulthard, C. E., Michaelis, R., Short, W. F., Sykes, G., Skrimshire, G. E. H., Standfast, A. F. B., Birkinshaw, J. H., and Raistrick, H., *Nature*, **150**, 634–35 (1942)
256. Waksman, S. A., and Woodruff, H. B., *J. Bact.*, **44**, 373–84 (1942)
257. Roberts, E. C., Cain, C. K., Muir, R. D., Reithel, F. J., Gaby, W. L., Van Bruggen, J. T., Homan, D. M., Katzman, P. A., Jones, L. R., and Doisy, E. A., *J. Biol. Chem.*, **147**, 47–58 (1943)
258. Birkinshaw, J. H., and Raistrick, H., *J. Biol. Chem.*, **148**, 459–60 (1943)
259. Van Bruggen, J. R., Reithel, F. J., Cain, C. K., Katzman, P. A., Doisy, E. A., Muir, R. D., Roberts, E. C., Gaby, W. L., Homan, D. M., and Jones, L. R., *J. Biol. Chem.*, **148**, 365–78 (1943)
260. Schales, O., *Arch. Biochem.*, **2**, 487–90 (1943)
261. Lipmann, F., and Owen, C. R., *Science*, **98**, 246–48 (1943)
262. Green, D. E., and Pauli, R., *Proc. Soc. Exptl. Biol. Med.*, **54**, 148–50 (1943)
263. Weindling, R., *Phytopathology*, **31**, 991–1003 (1941)
264. Johnson, J. R., Bruce, W. F., and Dutcher, J. D., *J. Am. Chem. Soc.*, **65**, 2005–9 (1943)

265. Chain, E., Florey, H. W., Jennings, M. A., and Williams, T. I., *Brit. J. Exptl. Path.*, **24**, 108–19 (1943)
266. Duffin, W. M., and Smith, S., *Nature*, **151**, 251 (1943)
267. Abraham, E. P., Chain, E., Baker, W., and Robinson, R., *Nature*, **151**, 107 (1943)
268. McIlwain, H., *Biochem. J.*, **37**, 265–71 (1943)
269. McIlwain, H., *J. Chem. Soc.*, 322–25 (1943)
270. Woolley, D. W., and McCarter, J. R., *Proc. Soc. Exptl. Biol. Med.*, **45**, 357–60 (1940)
271. Dam, H., Glavind, J., Orla Jensen, S., and Orla Jensen, A. D., *Naturwissenschaften*, **29**, 287–88 (1941)
272. Kumler, W. D., and Daniels, T. C., *J. Am. Chem. Soc.*, **65**, 2190–96 (1943)
273. Waksman, S. A., and Tishler, N., *J. Biol. Chem.*, **142**, 519–28 (1942)
274. Waksman, S. A., and Woodruff, H. B., *J. Bact.*, **44**, 373–84 (1942)
275. Foster, J. W., and Woodruff, H. B., *Arch. Biochem.*, **3**, 241–56 (1943)
276. Waksman, S. A., and Bugie, E., *Proc. Soc. Exptl. Biol. Med.*, **54**, 79–82 (1943)
277. Ungar, J., *Nature*, **152**, 245–46 (1943)
278. Turner, J. C., Heath, F. K., and Magasanik, B., *Nature*, **152**, 326 (1943)
279. Foster, J. W., and Wilker, B. L., *J. Bact.*, **46**, 377–89 (1943)
280. Mayer, R. L., *Science*, **98**, 203–4 (1943)
281. Waksman, S. A., *J. Bact.*, **46**, 299–310 (1943)
282. Woodruff, H. B., and Foster, J. W., *Arch. Biochem.*, **2**, 301–16 (1943)
283. Dubos, R., *J. Exptl. Med.*, **70**, 1–10 (1939)
284. Clutterbuck, P. W., Lovell, R., and Raistrick, H., *Biochem. J.*, **26**, 1907–18 (1932)
285. Jones, H., Rake, G., and Hamre, D. M., *J. Bact.*, **45**, 461–69 (1943)
286. Scott-Moncrieff, R., *J. Genetics*, **32**, 117–70 (1936)
287. Birkinshaw, J. H., and Raistrick, H., *Biochem. J.*, **28**, 828–36 (1934)
288. Clutterbuck, P. W., Raistrick, H., and Rintoul, M. L., *Phil. Trans. Roy. Soc. London*, B., **220**, 301–30 (1931)
289. Oxford, A. E., and Raistrick, H., *Biochem. J.*, **26**, 1902–6 (1932)
290. Oxford, A. E., and Raistrick, H., *Biochem. J.*, **34**, 790–803 (1940)
291. Foster, J. W., Carson, S. F., Ruben, S., and Kamen, M. D., *Proc. Natl. Acad. Sci. U.S.*, **27**, 590–96 (1941)
292. Krebs, H. A., *Ann. Rev. Biochem.*, **12**, 529–50 (1943)
293. Commoner, B., and Thimann, K. V., *J. Gen. Physiol.*, **24**, 279–96 (1941)
294. Mann, T., *Nature*, **151**, 619–20 (1943)
295. Wirth, J. C., and Nord, F. F., *Arch. Biochem.*, **1**, 143–63 (1942–43)
296. Goepfert, G. J., and Nord, F. F., *Arch. Biochem.*, **1**, 289–301 (1943)
297. Sciarini, L. J., Mull, R. P., Wirth, J. C., and Nord, F. F., *Proc. Natl. Acad. Sci. U.S.*, **29**, 121–26 (1943)
298. Gould, B. S., Tytell, A. A., and Jaffe, H., *J. Biol. Chem.*, **146**, 219–24 (1942)
299. Sciarini, L. J., and Nord, F. F., *Arch. Biochem.*, **3**, 261–68 (1943)
300. Wirth, J. C., and Nord, F. F., *Arch. Biochem.*, **2**, 463–68 (1943)
301. Nisikawa, H., *Bull. Agr. Chem. Soc. Japan*, **16**, 97–99 (1940)

302. Clutterbuck, P. W., Muhopadhyay, S. L., Oxford, A. E., and Raistrick, H., *Biochem. J.*, **34**, 664–77 (1940)
303. Anslow, W. K., and Raistrick, H., *Biochem. J.*, **34**, 1124–33 (1940)
304. Birkinshaw, J. H., Chambers, A. R., and Raistrick, H., *Biochem. J.*, **36**, 242–51 (1942)
305. Birkinshaw, J. H., and Bracken, A., *J. Chem. Soc.*, 368–70 (1942)
306. Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **149**, 295–300 (1943)
307. Peck, R. L., and Hauser, C. R., *J. Biol. Chem.*, **134**, 403–12 (1940)
308. Anderson, C. G., Haworth, W. N., Raistrick, H., and Stacey, M., *Biochem. J.*, **33**, 272–79 (1939)
309. Albericci, V. J., Curtin, T. P., and Reilly, D., *Biochem. J.*, **37**, 243–46 (1943)
310. Hassid, W. Z., Baker, E. E., and McCready, R. M., *J. Biol. Chem.*, **149**, 303–11 (1943)
311. Starkey, R. L., and Waksman, S. A., *J. Bact.*, **45**, 509–19 (1943)
312. Birkinshaw, J. H., Findlay, W. P. K., and Webb, R. A., *Biochem. J.*, **36**, 526–29 (1942)
313. Foster, J. W., and Woodruff, H. B., *J. Bact.*, **46**, 187–202 (1943)
314. Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, **241**, 177–89 (1941)
315. Foster, J. W., and Woodruff, H. B., *J. Biol. Chem.*, **148**, 723 (1943)
316. Foster, J. W., and Woodruff, H. B., *J. Bact.*, **45**, 408–9 (1943)
317. Foster, J. W., *J. Biol. Chem.*, **144**, 285–86 (1942)
318. Joslyn, D. A., *Science*, **99**, 21–22 (1944)
319. Rake, G., Jones, H., and McKee, C. M., *Proc. Soc. Exptl. Biol. Med.*, **52**, 136–38 (1943)
320. Wilson, V., *Nature*, **152**, 475–76 (1943)
321. Rake, G., and Jones, H., *Proc. Soc. Exptl. Biol. Med.*, **54**, 189–90 (1943)
322. Lawrence, C. A., *Science*, **98**, 413 (1943)
323. Lawrence, C. A., *Science*, **99**, 15–16 (1944)
324. Stanley, A. R., *Science*, **99**, 59 (1944)
325. Tauber, H., Laufer, S., and Goll, M., *J. Am. Chem. Soc.*, **64**, 2228–29 (1942)
326. Clifton, C. E., *Science*, **98**, 69–70 (1943)
327. Robinson, G. H., and Wallace, J. E., *Science*, **98**, 329–30 (1943)
328. Meyer, K., Hobby, G. L., and Chaffee, E., *Science*, **97**, 205–6 (1943)
329. Meyer, K., Hobby, G. L., and Dawson, M. H., *Proc. Soc. Exptl. Biol. Med.*, **53**, 100–4 (1943)
330. Ryan, F. J., Beadle, G. W., and Tatum, E. L., *Am. J. Botany*, **30**, 784–99 (1943)
331. Ryan, F. J., and Brand, E., *J. Biol. Chem.* (In press)
332. Thompson, R. C., Isbell, E. R., and Mitchell, H. K., *J. Biol. Chem.*, **148**, 281–88 (1943)
333. Bonner, J., and Dorland, R., *Arch. Biochem.*, **2**, 451–62 (1943)

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HISTOCHEMISTRY

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INTRODUCTION

Histochemistry is still in that early stage of development where attention has been, of necessity, focused largely on techniques and methods that can be brought to bear toward the solution of its problems. The applications of the tools already acquired, as would be expected in a broad field, have followed highly diverse paths. While, admittedly, it would be preferable to base a review on the evolution of the applications, in the present case there are few common threads that can be followed. Hence, in the hope of achieving greater clarity and homogeneity, the reviewer has chosen rather to discuss the field from the point of view of the particular techniques that have been elaborated, and to treat together the applications of each general technique.

Since histochemistry has not been the subject of a previous review in these volumes, the author has considered it desirable to present more than an "annual" review in order to furnish a more complete and integrated picture of this young and rapidly growing border-science.

STAINING TECHNIQUES AND APPLICATIONS

In contrast to the extensive development and use of staining reactions for purely morphological studies of tissues and cells, the identification of chemical components by staining is still in a relatively primitive state. Since the usefulness of this technique is limited for the most part to qualitative detection, its importance might be underestimated; however, it often serves, as no other technique can at present, to enable localization of chemical components in tissues whose cell types or cytological parts cannot be adequately separated. In the case of a few compounds such as glycogen, calcium salts, etc., staining procedures have been employed for many years in routine histological examinations and these need not be discussed here.

Ascorbic acid.—The only vitamin for which a staining reaction has been extensively used is ascorbic acid. The stain depends on the deposition of reduced silver when the ascorbic acid in the tissue

interacts with acid silver nitrate; and its chief proponents have been Giroud & Leblond who applied the technique to numerous histological and cytological studies (1, 2, 3). In answer to many criticisms of the silver stain, the specificity of which for ascorbic acid has been questioned and the ability of which to detect this compound in certain tissues fails even though the vitamin is present, Giroud & Leblond claim that a positive reaction is a specific test for the substance but admit that the failure of a tissue to give the stain does not necessarily mean the absence of ascorbic acid (4).

Recently, Barnett & Bourne (5) employed the stain to observe the distribution of ascorbic acid in the developing chick from the fourth day of incubation to two days after hatching. Their results were in accord with earlier findings that demonstrated localization of the vitamin in the Golgi material of cells. Working with frogs and teleosts, Bargmann (6) found that when ascorbic acid was administered parenterally it could be demonstrated in the Golgi apparatus of liver cells.

Enzymes.—Direct microscopic detection of a few enzymes by the use of color reactions has been made possible. Linderstrøm-Lang & Holter (7) have outlined the tests for peroxidase, phenolase, tyrosinase, and dopaoxidase, the "peptolytic enzyme" of Abderhalden, and the "oxydo-reductase LM" of Hirsch & Buchman.

More recently tissue stains for phosphatases have been developed and their applications already indicate that they can be expected to prove quite important. Independently and simultaneously, by a truly rare coincidence, Gomori (8) in Chicago and Takamatsu (9) in Japan developed the same stain for alkaline phosphatase based on the fact that the enzyme can withstand alcohol fixation and tissue embedding so that, when microtome sections are placed in a buffered substrate solution containing calcium ion, the enzymatic liberation of phosphoric acid is made apparent by precipitation of calcium phosphate at the sites of activity. Application of a calcium stain serves to visualize the precipitate. The solubility of calcium phosphate in the lower pH range makes it impossible to employ this procedure for acid phosphatase; hence, for this enzyme Gomori (10) used a lead phosphate precipitate which could be made to yield a brown or black stain by conversion to the sulfide.

Takeuchi & Takamatsu (11) studied alkaline phosphatase in tuberculous tissue and Gomori (12) investigated the distribution of the enzyme in normal organs and tissues. Later he (10) made a

similar study of acid phosphatase distribution. The latter was found to be present in particularly high concentrations in the spleens of a variety of species, the human prostate, and certain malignant epithelial tumors. Huggins *et al.* (13) applied the stain for acid phosphatase to studies of the effect of castration on prostatic carcinoma. Meanwhile Kabat & Furth (14) demonstrated that alkaline phosphatase is particularly characteristic of the normal cells of the epithelium of the small intestine and of proximal convoluted tubules, osteoblasts, and endothelium. It was shown that the enzyme is conspicuous in various osteogenic malignancies but absent in certain non-bone-forming types. Subsequently Landow, Kabat, & Newman (15) investigated this enzyme in the nervous systems of normal and pathological animals, and of man. Bourne (16) employed the same stain with some modification to elucidate the distribution of alkaline phosphatase in regenerating bone. He found that lessened activity occurred in the periosteum and regenerating bone of scorbutic animals and demonstrated that the enzyme does not appear to be associated with calcification of the chicken egg although it plays some part in the secretion of the shell of certain molluscs.

Moog (17) found both acid and alkaline phosphatases in the spinal cord of the embryonic chick. While the alkaline phosphatase was more active during all stages of development, both enzymes could be detected in fairly high concentration in the neural tissue as early as the first day of incubation. During development the alkaline phosphatase becomes localized in the white matter throughout; the acid enzyme becomes concentrated in the motor groups and the ventral half of the cord. Nuclear membranes and all nucleoli appear to contain alkaline phosphatase, but nuclei do not show the acid phosphate reaction. Later Moog (18) found that manganous sulfate could activate the acid phosphatase reaction and thus bring about a darkening of the pale stain produced in tissues poor in the enzyme.

Further improvements have appeared lately by Gomori (19) who made possible the visualization of insoluble calcium salts, preexisting in the tissue, by converting them to a black precipitate of cobalt sulfide, and of calcium phosphate resulting from phosphatase activity by staining it a purplish red. He applied this procedure to a study of calcification in normal and pathological tissue and showed that phosphatase is involved in calcification of living tissue but not in that of hyaline connective tissue (19).

An extensive study of conditions required to yield maximal enzyme

activity was recently reported by Wolf, Kabat & Newman (20) with the result that the enzyme could be demonstrated in tissues that had given negative stains heretofore. These workers applied their modified Gomori method to an investigation of acid phosphatase in normal and neoplastic tissues of the nervous system (20).

Before leaving the subject, attention should be called to the interesting work of Krugelis (21) who presented evidence for the occurrence of alkaline phosphatase in the chromosomes of mouse testes.

The histochemical demonstration of amine oxidase has been accomplished by Oster & Schlossman (22) who utilized the fact that the aldehydes formed by action of this enzyme may be visualized by means of fuchsin sulfurous acid. Naturally occurring "plasmal" aldehyde was first bound with bisulfite to prevent interference with the detection of aldehyde formed enzymatically when fresh frozen sections of tissue were incubated with tyramine in phosphate buffer at pH 7.2. These authors found that amine oxidase was localized in the distal convoluted tubules of guinea pig kidney and thus attributed a new function to this portion of the nephron previously considered chiefly an organ of absorption. Substitution of *l*-tyrosine for tyramine indicated the presence of a decarboxylase in the same histological site as the amine oxidase. Decarboxylation of the tyrosine is believed to precede the oxidation of its amino group in the kidney.

Hormones.—Bennett (23, 24) developed a histochemical method for the demonstration of ketosteroid hormone in the adrenal cortex but Gomori (25) showed that this test is not specific. The test depends on the formation of a yellow phenylhydrazone when frozen sections are treated with phenylhydrazine, so that it merely indicates the presence of lipids having aldehyde or keto groups, which would include Feulgen's "plasmal."

Nucleic acid.—Nucleic acid has been identified in tissues by the Feulgen reaction but, as in the case of the silver stain for ascorbic acid, a negative result does not exclude the presence of the substance (26). A positive color reaction is believed to demonstrate desoxyribonucleic acid (27). Kelley (28) investigated the mechanism of basic dye staining of nucleoproteins. The use of ultraviolet absorption methods for detection of nucleic acid will be considered later.

Bile constituents.—A staining reaction for bile pigments and salts, depending on precipitation with barium ion followed by an acid fuchsin stain, was developed by a group of Swedish investigators in the course of studies on liver function (29 to 32). A reaction for the

histochemical demonstration of bilirubin has been reported by Stein (33).

Acid.—Although neutral red has been used in studies of acid secretion in the stomach (34), the results have been unsatisfactory and inconclusive as borne out by the work of Lison (35, 36) and Gersh (37). An exhaustive and critical discussion of attempts to elucidate the mechanism of acid formation in the stomach has been given by Hollander (38).

Sulfonamides.—The present great interest in the sulfa drugs lends particular importance to the technique of MacKee *et al.* (39) for the demonstration of these compounds in tissues. The fresh tissue is fixed with dry formaldehyde gas, and after sectioning on a freezing microtome, the slices are treated with *p*-dimethylaminobenzaldehyde in acid-alcohol solution in order to visualize the sulfa compounds as yellow to orange precipitates. Air produces a bleaching effect which is obviated by mounting the sections in dammar resin. Applications of this staining method should produce results of considerable value. Already MacKee *et al.* (40) have investigated the penetration of sulfa drugs into skin.

MICROBIOLOGICAL TECHNIQUES AND APPLICATIONS

The effects of a number of biologically important substances on the metabolism of certain yeasts and bacteria have been employed as a means of assaying many of these substances. The review last year by Van Niel (41) gives the later references to these assay methods which have dealt particularly with vitamins of the B family.

A few adaptations of the microbiological technique to histochemical studies have begun to appear. Metcalf & Patton (42) determined the riboflavin in the Malpighian tubes of the American roach in the course of a study on the fluorescence microscopy of this insect. Earlier, Drilhon & Busnel (43) had obtained evidence of a flavin compound in the Malpighian tubes and followed this with a report that Malpighian cells from the venom glands of Hymenoptera contained 240 to 670 μg. riboflavin per gm. (44).

Bonner & Dorland (45) determined the riboflavin and pantothenic acid in tomato plants. They observed that both vitamins exist in higher concentrations in the apex and young leaves of the plant than in the base and older leaves. Pantothenic acid was found to accumulate above the basal girdle made by steaming the plant at the second node. Also,

an accumulation of this vitamin was noted on the laminar side of the girdled petioles of mature leaves. Riboflavin did not accumulate significantly above girdles on the stem base or petioles.

A preliminary study of the B vitamins in the nuclei of the cells of beef heart and mouse mammary carcinoma was reported by Isbell *et al.* (46), and other studies by the University of Texas group include changes in the content of B vitamins in tissues as a function of embryonic development (47) and of later stages of development (48).

CHEMICAL TECHNIQUES AND APPLICATIONS

Colorimetric Investigations

Colorimetry has found little place in histochemical studies since in most cases the more quantitative titrimetric or gasometric procedures can be employed with equal or greater facility. However, in a few instances, colorimetric methods have offered the advantage of ease or simplicity of operation while retaining sufficient accuracy. Thus phosphorus and phosphatase in 50 c.mm. blood samples were determined by Lundsteen & Vermehren (49) who used a step-photometer with a microcuvette having a capacity of about 0.2 cc. Levy (50) employed this instrument in his Kjeldahl method which he used to measure the nitrogen in microtome sections of barley grains. This method is suitable for quantities of nitrogen from 0.5 to 6 μg. The step-photometer was also adapted to the estimation of lactic acid in the range of 2 to 10 μg. by Miller & Muntz (51) in their work on carious tooth substance, serum, and saliva.

The most recent of the series of notable papers from Richard's laboratory on the quantitative study of the composition of glomerular urine is the report by Bott (52) of the concentration of sodium in the glomerular urine of *Necturi*. By the method described, sodium can be determined with an accuracy of about ±3 per cent in samples as small as 0.2 c.mm. which contain as little as 0.3 μg. of sodium. Bott observed that the sodium concentration of the glomerular fluid is within 5 per cent of that in the serum, thus contributing more evidence that indicates glomerular fluid is an ultrafiltrate of plasma.

Titrimetric Investigations

Linderstrøm-Lang–Holter technique.—The elaboration of titrimetric procedures and their application to histochemical problems have resulted very largely from the classical work of Linderstrøm-

Lang & Holter which began in 1931. Their burettes divided in 0.2 c.mm. graduations, their fine pipettes and magnetic stirring method, and their manner of handling individual microtome sections and cells have led to the development, by them and their collaborators, of procedures for the determination of a variety of enzymes, elements, and compounds of biological importance. At the present time the methods include measurements of protease, peptidases, pepsin, trypsin, urease and other amidases, arginase, carbohydrases, catalase, esterases and lipase, calcium, nitrogen, potassium, acid, alkali, glycogen, reducing sugars, chloride, amino groups, carboxyl groups, ammonia, and ascorbic acid. These measurements can be carried out on separate microtome sections, isolated cells such as the sea urchin egg, and cell fragments. A number of reviews describing these methods and their applications have appeared (7, 53, 54, 55).

More recent applications of the technique comprise a rather diverse group of studies. Avery & Linderstrøm-Lang (56) investigated the distribution of peptidase in the *Avena* coleoptile (phytohormone test object). They found that for coleoptiles of 4 mm. or more in length a greater enzyme activity per unit weight and per cell exists at the tip, and that an approximate parallelism maintains between the peptidase and auxin gradients.

Further work on the histochemistry of the stomach by Søeborg-Ohlsen (57) showed that in the hog stomach dipeptidase predominates in the epithelium, while aminopolypeptidase and pepsin appear mainly in the chief-cell region of the fundus. Cathepsin activity was negligible throughout the stomach and no differences in enzyme activities were observed between the full and empty organs. In the case of human stomach tissue removed from patients with duodenal ulcers, the dipeptidase and aminopolypeptidase activities showed the same distribution as in hog stomach. Also in the human tissue slight pepsin activity was observed in the pylorus but a great deal was found in the fundus, and considerable urease was demonstrated to be present in the mucosal surface of the pylorus.

A group of papers on the histochemistry of the adrenal gland has appeared. In one of these papers the distribution of cholinesterase was described (58). The enzyme was found to occur in greater concentration in the medulla.

Weil & Russell (59) made a study of plasma phosphatase in relation to fat metabolism in rats. Weil & Jennings (60) have extended earlier work on the histochemistry of the rabbit kidney to include the

distribution of cathepsin, aminopolypeptidase, and esterase. "Cells of the proximal and distal convoluted tubules were about twice as active enzymatically as the cells of the ascending and descending tubules of Henle's loop and about 4 times as active as the cells of the collecting tubules." Dipeptidase showed essentially the same distribution although no activity was observed in cells of the collecting tubules. Amylase activity was associated with the cells of the proximal and distal convoluted tubules and collecting tubules but none was found in the loops of Henle.

In a study of retinal histochemistry, Anfinsen, Lowry & Hastings (61) showed that the usual method of employing alternate sections for chemical determination and histological examination could not be used because of structural changes that occur every 40 to 50 μ. Hence they developed a technique in which the same section could be stained for histological study and subsequently used for enzyme measurement. This is the reverse of the procedure used by Glick & Biskind in earlier work on the histochemistry of vitamin C in which staining was applied after chemical determination on the same section. Anfinsen *et al.* rapidly froze the tissue in isopentane cooled by liquid nitrogen, and, after trimming to a block, immersed it in a mixture of dry ice and petroleum ether until it was transferred to a microtome kept in a cryostat at —20° according to the microtome technique of Linderstrøm-Lang & Mogensen (62). The sections, cut at 20 μ, were dehydrated at —20° with P_2O_5. Methyl violet was used to stain the cytoplasm. The xylol medium was removed, and, after air drying, the sections were ready for chemical measurement. This technique exerted no significant effect on the peptidase or diphosphopyridine nucleotide in rat liver or the cholinesterase in rat brain cortex.

The Kjeldahl method for nitrogen which Levy had previously developed was modified by Levy & Palmer (63) so as to eliminate distillation. The method is based on the reaction of ammonia with hypobromite. The authors, in a series of papers on the chick embryo, explored the kinetics of dipeptidase action in embryo extracts (64), the accumulation of mass, nitrogen, and dipeptidase in embryos of from one and one-half to eighteen days incubation (65), and the distribution of dipeptidase in the cephalic region of the three-day embryo (66). More recently (67) the accumulation of aminopeptidase during development of the chick embryo was followed. It was also demonstrated that glycylglycyl-*d*-alanine, in which the carboxyl group is carried by the residue of an unnatural amino acid, is hydrolyzed by

the embryo extract from the amino end only. Holter & Lindahl (68) studied the distribution of peptidase in embryos of *Paracentrotus lividus* during the first sixty hours of development. The enzyme followed the cytoplasm distribution and selective accumulation in the embryo could not be demonstrated.

Pickford (69) carried out a thorough investigation of the distribution of peptidase in the salamander gastrula. She observed that *l*-leucylglycine was hydrolyzed more rapidly than glycylglycine while *dl*-alanylglycine was split still more rapidly. The *d*-isomer of the latter substrate was not hydrolyzed enzymatically and a possible explanation of this fact, based on the increase of pH accompanying hydrolysis despite buffering, was put forward (70). This explanation will probably prove faulty however, since, as Levy (71) has pointed out, the pH optima are not particularly sharp and the calculated pH change is not sufficient to abolish activity; furthermore, the possibility that the optical specificity of peptidases from various sources is different has not been considered by Pickford.

The constancy of dipeptidase activity in the ciliate, *Didinium nasutum,* undergoing division and deprived of food, was demonstrated by Doyle & Patterson (72). The parent had exactly twice the enzyme of each of its daughter cells and four times that of each cell formed by the succeeding division. When a paramecium was fed to a didinium the enzyme content was found to remain the sum of that of the predator and prey throughout the ensuing four hour period of observation. This period is approximately the duration of one cell-generation.

Histochemical studies of the liver of the rat from sixteen to thirty days after the beginning of gestation were reported by Dumm (73). Changes in the chloride, various forms of phosphorus, glycogen, solids, and peptidase were measured, and these data were correlated with the changes in the extracellular and intracellular phases.

Sawyer (74) followed the development of cholinesterase throughout the larval life of *Amblystoma punctatum* and observed a close correlation between the enzyme activity and function. When larvae were reared in solutions of cholinesterase inhibitors, both enzyme activity and physiological reactions could be correlated, and on removal from the inhibitor solutions the recovery of both was parallel. The author concludes that cholinesterase action is a biochemical criterion of functional capacity in neuromuscular apparatus, and that the physiological development of this system can be assayed in terms of the enzyme activity. The reviewer should like to inject the caution that drugs

having powerful inhibiting effects on cholinesterase also exert pharmacological reactions that cannot be explained solely by these effects and seem, in fact, to be independent of them. For this reason the possibility should be borne in mind that cholinesterase activity may not be the only etiological factor involved in the physiological responses observed, even though a parallelism may exist between the enzyme activity and these responses.

A correlation between cholinesterase activity and neuroblast differentiation was observed by Tahmisian (75) who studied the enzyme activity of extracts of eggs of *Melanoplus differentialis* daily throughout their embryonic life. The pH and temperature optima found for these grasshopper eggs were similar to the optima reported for mammalian sources. Enzymogenesis was first apparent on the seventh day of pre-diapause and continued until the onset of diapause, when it ceased. It was resumed during post-diapause at which stage acetylcholine could be detected in the embryo for the first time.

Kirk technique.—Two years after the first of the series of histochemical publications by Linderstrøm-Lang & Holter appeared, the earliest of a group of papers by Kirk and collaborators was published that dealt with methods and applications of "quantitative drop analysis." Although developed independently, the volumetric microtechnique employed was similar to that of the Danish scientists, and references to the procedures for estimation of calcium, nitrogen, iron, phosphorus, and reducing sugars may be found in a review by Kirk (76). Improvements in certain of these methods and various applications were considered in a subsequent review (77). Since the appearance of the latter publication, papers on the formol titration (78), potentiometric determination of chloride (79), and estimation of potassium (80) have been published. A report appeared on the chemical metabolism of *Paramecium caudatum* in which certain of the methods were used to follow the formation of acid and the nature of the nitrogenous excretion products under various nutritional conditions (81).

Conway diffusion apparatus.—For the determination of minute quantities of volatile compounds such as ammonia, special diffusion cells have been developed, chiefly by Conway (82), in which the volatile constituent liberated from one solution can be absorbed in a separate solution within the same cell. This apparatus has been applied to the measurement of ammonia, urea, chloride, and, most recently, glucose (83). The same end has been accomplished in a somewhat simpler manner in certain of the Danish methods (7).

Other techniques.—Employing features of the Linderstrøm-Lang-Holter technique and the Conway diffusion method, Borsook & Dubnoff (84) developed electrometric methods for the determination of total nitrogen, ammonia, amino and amide nitrogen, peptides, adenylic acid, and nitrates, which they applied to an investigation of the nitrogen metabolism of certain plant and animal tissues.

A modified Wigglesworth micromethod for chloride was used by Dean (85) to show that isolated muscle fibers of frog contained an average of 15×10^{-6} moles of chloride per cc., with most of the chloride localized on the surface of the fibers. This finding, it was pointed out, is compatible with the view that the muscle fiber membrane is impermeable to chloride. Although the procedure employed served the investigator well, it should be mentioned that this method lacks certain of the technical refinements and advantages inherent in the electrometric microdetermination developed by Linderstrøm-Lang, Palmer & Holter (86), or the similar procedure reported by Cunningham, Kirk & Brooks (79).

Before leaving this section, attention should be called to Lowry's clever quartz capillary spring balance (87), of particular interest for histochemical work. It has a precision of ± 0.1 µg. with a maximum load of 0.3 mg. Lowry employed the instrument for the study of the distribution of dry matter and fat in the gastric mucosa of the hog.

Gasometric Investigations

Many histochemical problems require gasometric investigations; however, the techniques available in the past have been, for the most part, not sufficiently delicate to permit studies on the minute bits of material it is necessary to employ. Fortunately, there has been a notable advance within recent years in the development of gasometric methods applicable to histochemical problems, and a few applications of these have already been forthcoming. A review of microrespiration techniques was published by Tobias (88) this year.

Cartesian diver micromanometer.—One of the newer methods that is certain to be widely adopted and applied is the ingenious Cartesian diver ultramicromanometer originated by Linderstrøm-Lang (89, 90). The instrument is about 1500 times as sensitive as the Warburg manometer. The reaction chamber usually employed has a volume of about 10 c.mm., and it is possible to measure gas changes down to 0.001 c.mm. since the volume change corresponding to 1 cm. on the manometer scale is in the range of from 0.008 to 0.022 c.mm.

The Cambridge group immediately appreciated the potentialities of this instrument and soon Boell, Needham & Rogers (91) introduced a few technical modifications and employed the apparatus for the study of the anaerobic glycolysis of the regions of the amphibian gastrula. Pieces of the embryo of about 100 μg. dry weight were employed and methods of dealing with such small amounts of tissue had to be evolved. From this work, it was learned that the dorsal blastopore lip region had three times the anaerobic glycolysis and ammonia production of the ventral ectoderm. Boell & Needham (92) continued this investigation by demonstrating that dinitro-*o*-cresol increases the anaerobic glycolytic rate and the effect is more pronounced in the ventral ectoderm than in the dorsal lip region. Subsequently, these workers (93) extended their studies to include respiration measurements, and with Koch (94) they determined respiratory quotients of the embryonic regions.

Meanwhile Lindahl & Holter (95), continuing the study of *Paracentrotus lividus* embryos previously noted (68), were investigating the respiration of the animal and vegetative halves of these embryos. They observed that the oxygen uptakes of the two halves were equal, and their sum equivalent to that of the whole embryo. The oxygen consumption per embryo per hour was in the range of from 2.8 to 7.5×10^{-4} c.mm., while that of unfertilized eggs was from 0.7 to 1.2×10^{-4} c.mm. per egg per hour.

Later Boell & Woodruff (96) studied the respiration of single *Paramecium calkinsi* in the Cartesian diver, and showed that the average oxygen consumption was 4.3×10^{-4} c.mm. per hour for Type I, and 4.8×10^{-4} for Type II.

Procedures were also elaborated for the measurement of cholinesterase (90), thiamin, and cocarboxylase (97). The latter method enables estimation of as little as 5×10^{-4} μg. of thiamin and 5×10^{-5} μg. of cocarboxylase. The cholinesterase method was applied by Glick (98) to a study of the rate of destruction of acetylcholine by the superior cervical ganglion of the cat, and by Means (99) to an investigation of this enzyme in the tissues of adult *Melanoplus differentialis*. In the latter work, it was shown that the greatest activity is found in the nervous tissues, where it would be expected in accordance with the theory of the chemical transmission of nerve impulses. This study nicely complements that of Tahmisian (75), already mentioned, on the cholinesterase in the developing embryo of the same insect.

Optical lever microrespirometer.—Another type of microrespi-

rometer was developed by Heatley, Berenblum, & Chain (100) in which the respiration chamber is fitted with a wall of thin mica. A change of gas pressure within the chamber causes the mica to bulge and a compensating external pressure can be applied to restore the wall to its original position as indicated by an optical lever. Heatley (101) later described an apparatus in which up to six chambers could be used together. This type of instrument is only about 200 times as sensitive as the Warburg apparatus and hence lacks the refinement of the Cartesian diver manometer. While the Heatley instrument can perform all the types of measurement possible on a larger scale with the Barcroft or Warburg apparatus, it has the drawback of being rather complicated mechanically. Its usefulness, however, has been adequately demonstrated by Berenblum, Chain & Heatley (102) in a study of the metabolic activities of small amounts of surviving tissues.

Capillary microrespirometers.—Within a year, still another form of microrespirometer was reported by Cunningham & Kirk (103). This one is in effect a Barcroft differential manometer of greatly reduced size. The two chambers, which may have various capacities down to about 35 c.mm., are connected by a capillary of less than 16 mm. in length. Pressure changes are determined by the position of a droplet of kerosene within the capillary. The instrument can measure oxygen uptakes from 0.1 to 10 c.mm. per hour. Like the Heatley apparatus, it requires special mechanical construction. Barth & Kirk (104) later simplified the instrument. Cunningham & Kirk (105) modified the Kalmus open tube capillary respirometer in order to measure the oxygen consumption of single *Paramecium caudatum* cells.

Tyler & Berg (106) announced a capillary microrespirometer of the constant pressure type to be used for volume changes down to about 0.003 c.mm., and Tobias & Gerard (107) developed a much finer respirometer capable of measuring changes of from 5 to 10×10^{-4} c.mm. per min., and ten or more samples could be handled at one time. Tobias (108) has since produced an instrument employing an interferometer to measure pressure changes down to about 0.004 mm. of mercury.

Micrometer-burette respirometer.—The most recent and among the very best microrespirometers are those which have been developed by Scholander. These instruments employ a microburette (109, 110) that is particularly ingenious, extremely sensitive, and elegantly simple. An ordinary micrometer is used to displace the mercury in the reser-

voir of a Rehberg-type microburette. Employing the usual micrometer spindle, the instrument can be considered reliable to 0.1 c.mm. However, if the original spindle is replaced by a $\frac{1}{16}$ in. drill rod, the accuracy can be extended to 0.02 c.mm. Utilizing the micrometer burette, Scholander devised volumetric microrespirometers with sensitivities of from 0.33 to 0.010 c.mm. per hr. (111), and a micro gas-analyzer capable of the quantitative determination of the constituents in 10 c.mm. of respiratory gases to within 0.1 per cent (112). These techniques were applied, with modifications adapted for each investigation, to a study of the gas content of tissue (113), the oxygen consumption of dragonfly larvae and sand crabs (114), the estimation of nitrogen in 40 c.mm. samples of blood and saliva (115, 116), and carbon monoxide in blood (117). Later a series of papers appeared on the microgasometric determination of blood gases in which 40 c.mm. samples were employed for analysis of oxygen (118) or oxygen and carbon monoxide (119), 120 c.mm. samples for nitrogen (120), and 13 c.mm. samples for carbon dioxide (121).

While Scholander and co-workers have employed the microburette for gasometric measurements, its possible use in titrimetric procedures should be pointed out. In its present form, the fine Scholander microburette and that of Linderstrøm-Lang & Holter are about equally accurate. However, the principle of employing micrometer calibrations, as applied in the former instrument, should permit the construction of microburettes of greater sensitivity than is practicable in instruments of the latter type employing calibrated glass capillary tubes. The difficulties attending the use of very fine calibrated glass capillaries are obviated in the micrometer burette, the accuracy of which is independent of the glass bore and the adherence of solution to the capillary walls. The utilization of more sensitive micrometers, or drill rods of smaller diameter, or both, should enable significant refinement.

Polarographic microrespirometry will be considered later in the section on polarographic techniques.

Warburg and Barcroft techniques.—It should be pointed out that noteworthy histochemical studies have been carried out with the ordinary Warburg or Barcroft apparatus. The very extensive and excellent series of papers by Bodine and co-workers on the tyrosinase and protyrosinase in the developing grasshopper egg is a clear example. In the latest of these Bodine & Tahmisian (122) showed that the tyrosinase content of the parthenogenetic egg is about half that of the normal fertilized egg, and evidence was given which suggests that

this enzyme is produced by the serosa cells of the parthenogenetic embryo, just as had been shown previously to be the case in the normal embryo.

Flexner *et al.* (123) investigated the oxygen consumption, cytochrome, and cytochrome oxidase in relation to the histological structure of the developing cerebral cortex of the fetal pig. Two critical periods were observed: in the first, half way through gestation, cytochrome and its oxidase both increased significantly; in the second, four fifths of the way through gestation, the Q_{O_2} rose to the adult level. The Q_{O_2} of the chick embryo and its various tissues was determined by Romanoff (124) who found that, while the quotient for the whole embryo decreases with development, the rate in the anterior exceeds that in the posterior region during the period of predominant neurogenetic development, but this is reversed during organogenetic development.

The noteworthy contributions of Friedenwald, Herrmann, and coworkers on the histochemistry of the eye have already included the demonstration that a highly active cytochrome oxidase-succinodehydrogenase-fumarase system is confined almost entirely to the epithelial cells of the chorioid plexus while the malic and lactic dehydrogenases are distributed almost evenly between the epithelium and the stroma (125). However, greater cholinesterase activity was observed in the stroma than in the epithelium (126).

That respiration of the cartilage cell declines with advancing age while glycolysis does not change was shown by Rosenthal *et al.* (127). These workers also made a study of various dehydrogenases in bovine articular cartilage (128).

Continuing work mentioned earlier (56) on the *Avena* coleoptile, Berger & Avery made a study of dehydrogenases (129) in *Avena* coleoptile and demonstrated that synthetic auxins do not accelerate their actions (130). These authors subsequently brought out the interesting observation that alcohol dehydrogenase activity is stimulated by auxin treatment of plant tissue and suggested that this effect may be the basis of the mechanism of auxin action (131).

Deductive Methods

In certain instances it has been possible to obtain quantitative histochemical data from usual macrochemical analyses by what Lowry has termed "deductive histochemical methods." A clear and concise presentation of the principle of these methods has been given very recently

by Lowry (132) with a review of results obtained by such methods. The principle is well illustrated by Lowry's example:

> As a first approximation, a tissue such as skeletal muscle may be considered to be composed of 5 separate fractions, blood, fat, collagen plus elastin, extracellular fluid, and cells. If the amount of the first 4 fractions can be determined, the amount of the remaining intracellular fraction may be calculated. Furthermore, if one knows the composition of the blood and extracellular fluid, it becomes possible to calculate the concentration of a particular substance, A, in the cells by simply (1) measuring the total amount of A, (2) calculating the amount of A in the several extracellular fractions, (3) subtracting the extracellular A from the whole, and finally, (4) dividing the net intracellular A by the calculated amount of intracellular fraction. This is similar to the calculation of the concentration of chloride in red cells when the hematocrit and the concentration of chloride in whole blood and serum are known.

The chief applications of these methods have dealt with the study of electrolytes in cytoplasm. The noteworthy work of Lowry, Hastings, and collaborators (133 to 136) on the histochemistry of the aging process should be mentioned at this point. Dumm (73) has drawn on these methods in her study of histochemical changes in rat liver cited earlier.

PHYSICAL TECHNIQUES AND APPLICATIONS

Dilatometric Investigations

The fact that chemical reactions occurring in liquid phases are accompanied by isothermal changes in volume has been utilized in dilatometric methods to follow the course of reactions. We are indebted to the genius of Linderstrøm-Lang for the application of the dilatometric principle to the elaboration of a technique whereby the rates of reactions proceeding in aqueous droplets can be determined with a high degree of precision. In this manner, refinements over common macromethods of the order of 100,000 times were effected and the way opened for application to various histochemical studies. Cathetometer measurements of the displacements resulting from the density changes accompanying the reaction in a droplet suspended in a density gradient become a measure of the rate of the reaction. The density gradient is maintained in a bromobenzene-kerosene medium, and hence the technique cannot be used for reactions involving substances soluble in this medium.

The first reports of this dilatometric technique appeared in connection with an ultramicromethod for the estimation of peptidase activ-

ity (137, 138). Since the density measurement is accurate to about 3×10^{-6}, the hydrolysis of 0.3×10^{-10} mole of alanylglycine in a droplet of 0.1 c.mm. can be determined (approximately an amount of substrate that can be split by one sea-urchin egg in twenty seconds). The peptidase method was employed by Holter, Lanz & Linderstrøm-Lang (139) in a study of the localization of the enzyme during the first cell divisions of the *Psammechinus miliaris* embryo. They found that peptidase was distributed evenly among the eight blastomeres formed when the egg had undergone three divisions, and an even distribution maintained between the two halves of four blastomeres each. This investigation is related to the previous studies on *Paracentrotus lividus* (68, 95).

One of the chief difficulties in histochemical or cytochemical investigations is obtaining a quantitative definition of the structural elements in tissues or cells to which the quantities of an enzyme, or other substance measured, can be referred. In certain cases, it is advantageous to refer the measurements to the "reduced weight," a value independent of the water content of the sample. This value is defined as the weight of the sample minus the weight of an equal volume of water, and it can be determined with the gradient tube apparatus by placing the material in a drop of water and measuring the density of this drop (7). Then

$$\text{"reduced weight"} = (d - d_w)\, v$$

where d is the density measured, d_w is the density of water at the same temperature, and v is the volume of the drop.

Ultraviolet Microscopy

Absorption methods.—The range of usefulness of the microscopic examination of histological preparations has been broadened extensively through application of the ultraviolet quartz microscope with photographic or photoelectric attachments. As developed and applied by Caspersson (140), in particular, this technique has proved to be a beautiful means for the revelation of the nature and certain of the functions of the nucleic acids within the cell. The nucleic acids of chromosomes and other formed bodies have been characterized by their ultraviolet absorption curves by Caspersson and co-workers and reviews of these findings as well as their cytochemical significance have appeared. [Cf. Gersh (141) and Mirsky (142).] The striking advantage of the technique is that certain cell structures may be studied

in situ, and in absorption spectra measurements quantities of material down to 10^{-8} μg. will suffice. In one of the applications of this technique, organic iodine compounds as well as protein were identified in the thyroid colloid by Gersh & Caspersson (143). A modified apparatus, and its application to a histochemical analysis of changes in monkey motoneurons after root section, has been described by Gersh & Bodian (144). They made measurements of the extinction coefficients of the Nissl bodies in the nerve material, and were able to show that ribonuclease removes ribonucleotides from Nissl bodies, and that the decreased absorption of cytoplasm, caused by the enzyme, is probably the result of either nonspecific protein digestion or depolymerization of ribonucleoproteins, or both.

Fluorescence methods.—Popper (145) has succeeded in utilizing the fluorescence of vitamin A in ultraviolet light for the microscopic detection of this substance in tissue sections. Tissue fixed in 10 per cent formaldehyde is frozen and sectioned. When subjected to ultraviolet illumination under the microscope the vitamin A in the sections becomes apparent as a fading green fluorescence. The reliability of the method is indicated by the parallelism existing between observations of this nature and chemical determinations on the same material (146). The histological distribution of vitamin A in both normal and pathological human tissues (147), and in rat organs (148) was mapped out by Popper and co-workers. In a subsequent investigation (149) it was shown that vitamin A_1, which gives the quickly fading green fluorescence, could be differentiated from vitamin A_2, which is characterized by a slowly fading pale yellow-brown fluorescence. The A_1 is found in salt-water fish, while the A_2 appears in fresh-water varieties.

Popper and co-workers have been most productive in their applications of the fluorescence technique to a variety of physiological and pathological problems. These include the following: demonstration of vitamin A in the retina (150), human tumors (151), and human skin (152); its variation in the ovary during cyclic changes (153); its distribution in experimental liver damage (154); its change in choline deficiency (155); its fate during depletion (156); its metabolism in regard to hepatic function (157); and finally the relation between hepatic and plasma concentrations of the vitamin in humans (158).

Hirt & Wimmer have made a beginning toward the detection, in a similar manner, of nicotinic acid or its amide (159) and riboflavin

(160) in tissues. The former is made apparent by a stable yellow fluorescence and the latter by a green fluorescence. The riboflavin metabolism in the roach, previously noted (42), was investigated in this manner and evidence was presented for the existence of a bound form of riboflavin giving a yellow-orange fluorescence. Tests were made for the occurrence of the vitamin in the Malpighian tubes of a number of other insects as well.

In studies of carcinogenesis in mice elicited by 20-methylcholanthrene, Simpson & Cramer (161) employed the fluorescence of this substance to follow its distribution in the epidermis.

Electron Microscopy and Microincineration

It is only natural that a new tool is subjected to tests in every field where it may find use; so it has been with the electron microscope the uses of which in biology were reviewed last year by Marton (162). This instrument has yielded valuable information concerning the physical structure of cytological components, such as the findings of Schmitt *et al.* (163) on the ultrastructure of protoplasmic fibrils, but to date only Scott and co-workers have utilized electron microscopy for identification of substances in histological preparations.

For some time Scott has been employing microincineration of tissue sections, after freezing-drying, for the study of total inorganic salt distribution. With the electron microscope Scott & Packer (164) have been able to localize calcium and/or magnesium in the ash pattern of striated muscle; the elements cannot be differentiated by this method. The calcium and magnesium in sympathetic ganglion cells (165), and the minerals in epithelium of gastric mucosa (166) have also been studied. Cowdry's group have made similar investigations on hyperplasias of the mouse epidermis (167). Technical details for carrying out studies of this nature have been given by Packer & Scott (168), and a recent review on mineral distribution in cytoplasm has appeared (169).

Further adaptation of the electron microscope to histochemistry may be expected, particularly since it is now possible to prepare the very thin sections necessary for use with this instrument. By changing the mechanism on microtomes commonly used, Richards *et al.* (170) were able to obtain 0.1 μ sections of muscle. O'Brien & McKinley (171) have designed a new microtome that also cuts 0.1 μ sections and seems to be adaptable to many tissues.

Polarographic and Related Investigations

Carruthers has devised polarographic micromethods for sodium (172) and magnesium (173) that have been applied particularly to the study of carcinogenic changes in mouse epidermis (174). It was stated that the sodium method can be used for "mere traces." The magnesium method is capable of the estimation of from 68 to 100 μg. of the element with an error of from ± 1.8 to ± 2.8 per cent.

Microrespiration techniques employing the polarograph have been developed but the chief objection to them has been the effect that mercury, from the dropping electrode, might have on the biological systems. This difficulty has been overcome by Laitinen & Kolthoff (175, 176), who developed a method for the estimation of oxygen in solution using a revolving platinum wire microelectrode instead of the mercury type. Davies & Brink (177, 178) have also used the platinum microelectrode for respiration studies, and they have been able to follow minute by minute the respiration of 10 mg. of nerve tissue with a sensitivity of 0.001 c.mm. of oxygen. Another means of avoiding contact between mercury and the cells under examination is the double vessel designed by Selzer & Baumberger (179).

An investigation of the relation between respiration, protoplasmic streaming, and auxin transport in *Avena* coleoptiles was undertaken by DuBuy & Olson (180). The polarographic and manometric measurements of the respiration of myelocytes were compared by Warren (181) who made it clear that only in the former method are results free of solubility and diffusion factors, and it was emphasized that studies in the literature of the Pasteur effect have been carried out under conditions in which solubility and diffusion factors determined the rate of respiration. Beecher *et al.* (182) described a polarographic method for the determination of the oxygen content of small amounts of body fluids.

Mechanical Separation and Chemical Studies of Cellular Components

The instrument common to practically all work on the separation of cellular components is the high-speed centrifuge. The capacities of this important tool and the particulate components that have been isolated by its means have been the subject of a collection of excellent reviews (183) published in honor of Prof. R. R. Bensley. The point of view stressed by Bensley (184) that "to separate separable things

before proceeding to their analysis" has become an increasing tendency in cytochemical work. What follows will deal with recent chemical considerations of "separable things."

Isolation of cell nuclei.—Until recently, chemical studies on cell nuclei have been seriously handicapped by lack of means for isolating homogeneous material in sufficient quantities for chemical procedures. The old freezing-melting technique of Warburg resulted in damage to the nuclei and necessitated further separations. Crossmon (185) succeeded in separating the nuclei of muscle cells by the simple expedient of teasing a bit of tissue in 5 per cent citric acid which caused ejection of the nuclei from the cells. This technique was modified by the addition of pepsin [Stoneburg (186)] in order to obtain larger amounts of material, and was adapted for use on tumor and pus cells as well as on muscle; a report on the lipid analysis of the free nuclei was given. This method was not applicable to liver or thymus cells.

The acid treatment employed in the Stoneburg procedure denatures certain enzymes and proteins and hence is of limited value. Nevertheless, the technique was adequate for Marshak's (187) studies on the uptake of radioactive phosphorus, P_{32}, by nuclei. Marshak demonstrated that, if pepsin was omitted, the procedure of Stoneburg would permit the isolation of liver nuclei, and in this manner he could show that the nuclei of tumors accumulate more radioactive phosphorus, due to their mitotic activity, than do nuclei of liver cells. Mirsky & Pollister (188) used a modified Stoneburg method to obtain liver nuclei free of cytoplasm which they employed in studies on the fibrous nucleoprotein. The chemical properties of this substance lead to the conclusion that its chief, if not sole, cellular site of origin must be the nucleus.

The isolation of living nuclei from hen erythrocytes was accomplished by Laskowski (189) employing a different principle: he hemolyzed the cells with lysolecithin in neutral saline solution. Dounce & Lan (190) achieved the same end by using saponin which has advantages over lysolecithin of being more readily available and of not interfering with lipid studies. Later Dounce & Seibel (191) determined the acid phosphatase content of these nuclei.

The isolation of the nuclei of guinea pig liver cells was reported by Lazarow (192) who forced a fine liver suspension through bolting silk and separated the cellular components by subsequent centrifugation.

Within the past year Dounce (193) announced the preparation of rat liver nuclei by a process claimed to leave proteins and enzyme

systems undamaged. The frozen liver was added to a mixture of ice and citric acid solution, having a pH of from 6.0 to 6.2, in a Waring blendor. After straining the disintegrated tissue through cheese cloth, the liquid was centrifuged and the sediment washed a number of times. Nuclei prepared in this manner were used for studies of their content of arginase, catalase, cytochrome oxidase, esterase, dehydrogenases, phosphatases, cytochrome-*c*, coenzyme I, and riboflavin. Later Lan (194) investigated *d*-amino acid oxidase, uricase, and choline oxidase in rat liver nuclei obtained by the Dounce method. Then Dounce (195) showed that by isolating rat liver nuclei at pH 3.8 to 4.0 considerable protein denaturation occurs, but the greater acidity is favorable to retention of nucleic acid and protein by the nuclei. A study of the total lipid and desoxyribonucleic acid content of nuclei at pH 3.8 to 4.0 and at 6.0 to 6.2 indicated that there is a loss of protein in the latter case. The desoxyribonucleic acid content of isolated nuclei of tumor cells was also studied (196).

In his critical and masterly review of the methods of isolation of morphological constituents of the liver cell, Hoerr (197) has pointed out that Dounce's method of disintegrating tissue in a Waring blendor subjects the material to so severe a beating that the thixotropic property of the nucleoproteins is enhanced to the point of rapid gel formation. He has also indicated that Lazarow's procedure of chilling material quickly to 0°, but not allowing it to freeze, is preferable to the Dounce method and, in addition, avoids the excessive washings and centrifugings of the latter.

By means of sonic vibrations, Zittle & O'Dell (198) disintegrated bull spermatozoa so that on centrifugation heads, midpieces, and tails could be separated. Large variations were found in the lipid, sulfur, cystine, nitrogen, phosphorus, and nucleic acid content of these parts. Subsequently, Zittle & Zitin demonstrated that cytochrome oxidase (199) and iron (200) are greatest in the tails and least in the heads.

The separation of nuclei from embryos of rye was accomplished by Feulgen, *et al.* (201) by drying, grinding under benzene, suspending in a carbon tetrachloride-benzene mixture, and centrifuging. Later Behrens (202) isolated liver cell nuclei by dehydration of the frozen material followed by a procedure similar to that used for the rye germ; arginase and negligible lipase activity were demonstrated. Williamson & Gulick (203) determined calcium and magnesium in ashed thymus nuclei which had been isolated by dry grinding in the cold and centrifuging in a mixture of benzene and carbon tetrachloride. These

methods involving the use of organic solvents damage the nuclei and consequently are of limited application.

The well-known centrifugation technique of Harvey & Harvey has been applied particularly to the separation of egg cell fragments of *Arabacia punctulata*. More recent studies include measurements of oxidase activity (204), oxygen uptake (205), and, by means of the Cartesian diver, cytochrome oxidase (206).

Cytoplasmic components.—Although a number of cytoplasmic components have been isolated, it is still too early for much chemical work to have been done on them. The study of Nissl bodies *in situ* by ultraviolet absorption has already been discussed in this review.

Demonstration of succinodehydrogenase, cytochrome-*c*, and cytochrome oxidase in both mitochondria and the lipoprotein complex has been accomplished by Lazarow & Barron (207), while phosphatase was found in the lipoprotein by Kabat (208). Claude (209) has cited unpublished work of Hotchkiss & Hogeboom who showed that *d*-amino acid oxidase is localized in secretory granules of guinea pig and rat livers, and unpublished work of Woolley who found inositol in both microsomes and secretory granules, the quantities of inositol and lipid being directly proportional to one another. The separation of several macromolecular particles from cells such as iron-protein units, fowl tumor viruses, heavy conjugated proteins, and bacteriophage has been described by Stern (210). Lazarow (211) succeeded in separating particulate glycogen from guinea pig liver cells, and it was suggested that removal of glycogen from "solution" might shift the equilibrium of the reaction, glucose-1-phosphate $\rightleftharpoons$ glycogen + phosphate, to the right and thus initiate a series of changes related to glycogen storage and the regulation of blood sugar.

In the plant field, the isolation of chloroplasts by differential centrifugation has been of interest especially in relation to photosynthesis. Isolation studies have been carried out by Mesch (212), Granick (213), and Menke (214), and thus far analyses of protein, lipid, and ash have been given (214, 215, 216). Most of the catalase seems to be confined to the chloroplasts of leaf cells (215), and the production of oxygen by these bodies has been investigated by Hill & Scarisbrick (217). French & Anson (218) have made an extensive study of the influence of chemical and physical factors on the oxygen evolution.

LITERATURE CITED

1. GIROUD, A., AND LEBLOND, C. P., *L'Acide Ascorbique Dans Les Tissus Et Sa Détection* (Hermann et Cie, Paris, 1936)
2. GIROUD, A., LEBLOND, C. P., RATSIMAMANGA, R., AND RABINOWICZ, M., *Protoplasma,* **25**, 115–23 (1936)
3. GIROUD, A., *L'Acide Ascorbique Dans La Cellule Et Les Tissus* (Gebrüder Borntraeger, Berlin, 1938)
4. GIROUD, A., AND LEBLOND, C. P., *Anat. Record,* **68**, 113–26 (1937)
5. BARNETT, S. A., AND BOURNE, G., *Quart. J. Micro.-Sci.,* **83**, 259–99 (1942)
6. BARGMANN, W., *Zentr. inn. Med.,* **63**, 441–54 (1942)
7. LINDERSTRØM-LANG, K., AND HOLTER, H., "Die enzymatische Histochemie," in BAMANN, E., AND MYRBÄCK, K., *Die Methoden der Fermentforschung,* pp. 1132–62 (Thieme, Leipzig, 1940)
8. GOMORI, G., *Proc. Soc. Exptl. Biol. Med.,* **42**, 23–26 (1939)
9. TAKAMATSU, H., *Trans. Soc. Path. Japon.,* **29**, 492–98 (1939)
10. GOMORI, G., *Arch. Path.,* **32**, 189–99 (1941)
11. TAKEUCHI, T., AND TAKAMATSU, H., *Trans. Soc. Path. Japon.,* **29**, 490–92 (1939); **30**, 127–30 (1940)
12. GOMORI, G., *J. Cellular Comp. Physiol.,* **17**, 71–83 (1941)
13. HUGGINS, C., STEVENS, R. E., AND HODGES, C. V., *Arch. Surg.,* **43**, 209–23 (1941)
14. KABAT, E. A., AND FURTH, J., *Am. J. Path.,* **17**, 303–18 (1941)
15. LANDOW, H., KABAT, E. A., AND NEWMAN, W., *Arch. Neurol. Psychiat.,* **48**, 518–30 (1942)
16. BOURNE, G., *Quart. J. Exptl. Physiol.,* **32**, 1–19 (1943)
17. MOOG, F., *Proc. Natl. Acad. Sci. U.S.,* **29**, 176–83 (1943)
18. MOOG, F., *J. Cellular Comp. Physiol.,* **22**, 95–97 (1943)
19. GOMORI, G., *Am. J. Path.,* **19**, 197–209 (1943)
20. WOLF, A., KABAT, E. A., AND NEWMAN, W., *Am. J. Path.,* **19**, 423–40 (1943)
21. KRUGELIS, E. J., *J. Cellular Comp. Physiol.,* **19**, 376–79 (1942)
22. OSTER, K. A., AND SCHLOSSMAN, N. C., *J. Cellular Comp. Physiol.,* **20**, 373–78 (1942)
23. BENNETT, S. H., *Proc. Soc. Exptl. Biol. Med.,* **42**, 786–88 (1939)
24. BENNETT, S. H., *Am. J. Anat.,* **67**, 151–227 (1940)
25. GOMORI, G., *Proc. Soc. Exptl. Biol. Med.,* **51**, 133–34 (1942)
26. CASPERSSON, T., *Biochem. Z.,* **253**, 97–111 (1932)
27. LISON, L., *Histochemie Animale* (Gauthier-Villars, Paris, 1936)
28. KELLEY, E. G., *J. Biol. Chem.,* **127**, 55–71, 73–86 (1939)
29. FORSGREN, E., *Über die Rhythmik der Leberfunktion des Stoffwechsels und des Schlafes* (Marcus, Stockholm, 1935)
30. HOLMGREN, H., *Z. mikroskop.-anat. Forsch.,* **32**, 306–32 (1933)
31. HOLMQUIST, A. G., *Z. mikroskop.-anat. Forsch.,* **25**, 30–43 (1931)
32. HOLMQUIST, A. G., *Skand. Arch. Physiol.,* **65**, 9–17 (1932)
33. STEIN, J., *Compt. rend. soc. biol.,* **120**, 1136–38 (1935)
34. HOERR, N. L., *Anat. Record,* **65**, 417–35 (1936)
35. LISON, L., *Z. Zellforsch. mikroskop. Anat.,* **25**, 143–59 (1936)

36. Lison, L., *Protoplasma,* **24**, 453–65 (1935)
37. Gersh, I., *Proc. Soc. Exptl. Biol. Med.,* **38**, 70–72 (1938)
38. Hollander, F., *Gastroenterology,* **1**, 401–30 (1943)
39. MacKee, G. M., Herrmann, F., Baer, R. L., and Sulzberger, M. B., *Science,* **98**, 66–68 (1943)
40. MacKee, G. M., Herrmann, F., Baer, R. L., and Sulzberger, M. B., *J. Lab. Clin. Med.* (In press)
41. Van Niel, C. B., *Ann. Rev. Biochem.,* **12**, 551–86 (1943)
42. Metcalf, R. L., and Patton, R. L., *J. Cellular Comp. Physiol.,* **19**, 373–76 (1942)
43. Drilhon, A., and Busnel, R. G., *Compt. rend. acad. sci.,* **208**, 839–41 (1939)
44. Busnel, R. G., and Drilhon, A., *Compt. rend. soc. biol.,* **135**, 1008–9 (1941)
45. Bonner, J., and Dorland, R., *Am. J. Botany,* **30**, 414–18 (1943)
46. Isbell, E. R., Mitchell, H. K., Taylor, A., and Williams, R. J., "Studies on the vitamin content of tissues, II," *Univ. Texas Pub.,* No. **4237**, 81–83 (1942)
47. Taylor, A., Mitchell, H. K., and Pollack, M. A., "Studies on the vitamin content of tissues, I," *Univ. Texas Pub.,* No. **4137**, 67–80 (1941)
48. Williams, R. J., Taylor, A., and Cheldelin, V. H., "Studies on the vitamin content of tissues, I," *Univ. Texas Pub.,* No. **4137**, 61–66 (1941)
49. Lundsteen, E., and Vermehren, E., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **21**, 147–66 (1936) ; *Enzymologia,* **1**, 273–79 (1936)
50. Levy, M., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **21**, 101–10 (1936)
51. Miller, B. F., and Muntz, J. A., *J. Biol. Chem.,* **126**, 413–21 (1938)
52. Bott, P. A., *J. Biol. Chem.,* **147**, 653–61 (1943)
53. Linderstrøm-Lang, K., *Chem. Weekblad,* **36**, 4–12 (1939)
54. Linderstrøm-Lang, K., *Harvey Lect.,* Ser. **34**, 214–45 (1938–39)
55. Glick, D., *J. Chem. Education,* **16**, 68–76 (1939)
56. Avery, G. S., Jr., and Linderstrøm-Lang, K., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23**, 219–34 (1940) ; *Botan. Gaz.,* **102**, 50–63 (1940)
57. Søeborg-Ohlsen, A., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23**, 329–480 (1941)
58. Antopol, W., and Glick, D., *J. Biol. Chem.,* **132**, 669–73 (1940)
59. Weil, L., and Russell, M. A., *J. Biol. Chem.,* **136**, 9–23 (1940)
60. Weil, L., and Jennings, R. K., *J. Biol. Chem.,* **139**, 421–32 (1941)
61. Anfinsen, C. B., Lowry, O. H., and Hastings, A. B., *J. Cellular Comp. Physiol.,* **20**, 231–37 (1942)
62. Linderstrøm-Lang, K., and Mogensen, K. R., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23**, 27–34 (1938)
63. Levy, M., and Palmer, A. H., *J. Biol. Chem.,* **136**, 57–60 (1940)
64. Palmer, A. H., and Levy, M., *J. Biol. Chem.,* **136**, 407–13 (1940)
65. Levy, M., and Palmer, A. H., *J. Biol. Chem.,* **136**, 415–23 (1940)
66. Palmer, A. H., and Levy, M., *J. Biol. Chem.,* **136**, 629–35 (1940)
67. Levy, M., and Palmer, A. H., *J. Biol. Chem.,* **150**, 271–79 (1943)
68. Holter, H., and Lindahl, P. E., *Compt. rend. trav. lab. Carlsberg, Sér. chim.,* **23**, 249–56 (1940) ; *J. Cellular Comp. Physiol.,* **17**, 235–41 (1941)

69. Pickford, G. E., *J. Exptl. Zoöl.*, **92**, 143–70 (1943)
70. Pickford, G. E., *Science*, **98**, 14–15 (1943)
71. Levy, M. (Personal communication)
72. Doyle, W. L., and Patterson, E. K., *Science*, **95**, 206 (1942)
73. Dumm, M. E., *J. Cellular Comp. Physiol.*, **21**, 27–39 (1943)
74. Sawyer, C. H., *J. Exptl. Zoöl.*, **92**, 1–29 (1943)
75. Tahmisian, T. N., *J. Exptl. Zoöl.*, **92**, 199–213 (1943)
76. Kirk, P. L., *Ann. Rev. Biochem.*, **6**, 73–98 (1937)
77. Kirk, P. L., *Ann. Rev. Biochem.*, **9**, 593–616 (1940)
78. Sisco, R. C., Cunningham, B., and Kirk, P. L., *J. Biol. Chem.*, **139**, 1–10 (1941)
79. Cunningham, B., Kirk, P. L., and Brooks, S. C., *J. Biol. Chem.*, **139**, 11–19 (1941)
80. Cunningham, B., Kirk, P. L., and Brooks, S. C., *J. Biol. Chem.*, **139**, 21–28 (1941)
81. Cunningham, B., and Kirk, P. L., *J. Cellular Comp. Physiol.*, **18**, 299–316 (1941)
82. Conway, E. J., *Micro-Diffusion Analysis and Volumetric Error* (D. van Nostrand Co., New York, 1940)
83. O'Malley, E., Conway, E. J., and Fitzgerald, O., *Biochem. J.*, **37**, 278–81 (1943)
84. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **131**, 163–76 (1939)
85. Dean, R. B., *J. Biol. Chem.*, **137**, 113–21 (1941)
86. Linderstrøm-Lang, K., Palmer, A. H., and Holter, H., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **21**, 1–5 (1935)
87. Lowry, O. H., *J. Biol. Chem.*, **140**, 183–89 (1941)
88. Tobias, J. M., *Physiol. Revs.*, **23**, 51–75 (1943)
89. Linderstrøm-Lang, K., *Nature*, **140**, 108 (1937)
90. Linderstrøm-Lang, K., and Glick, D., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **22**, 300–6 (1938)
91. Boell, E. J., Needham, J., and Rogers, V., *Proc. Roy. Soc. (London), Ser. B*, **127**, 322–56 (1939)
92. Boell, E. J., and Needham, J., *Proc. Roy. Soc. (London), Ser. B*, **127**, 356–62 (1939)
93. Boell, E. J., and Needham, J., *Proc. Roy. Soc. (London), Ser. B*, **127**, 363–73 (1939)
94. Boell, E. J., Koch, H., and Needham, J., *Proc. Roy. Soc. (London), Ser. B*, **127**, 374–87 (1939)
95. Lindahl, P. E., and Holter, H., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **23**, 257–88 (1940)
96. Boell, E. J., and Woodruff, L., *Biol. Bull.*, **79**, 352–53 (1940)
97. Westerbrink, H. G. K., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **23**, 195–211 (1940); *Enzymologia*, **8**, 97–107 (1940)
98. Glick, D., *J. Gen. Physiol.*, **21**, 431–38 (1938)
99. Means, O. W., Jr., *J. Cellular Comp. Physiol.*, **20**, 319–24 (1942)
100. Heatley, N. G., Berenblum, I., and Chain, E., *Biochem. J.*, **33**, 53–67 (1939)
101. Heatley, N. G., *J. Sci. Instruments*, **17**, 197–202 (1940)

102. Berenblum, I., Chain, E., and Heatley, N. G., *Biochem. J.*, **33**, 68–74 (1939)
103. Cunningham, B., and Kirk, P. L., *J. Gen. Physiol.*, **24**, 135–49 (1940)
104. Barth, L., and Kirk, P. L., *J. Gen. Physiol.*, **25**, 663–68 (1942)
105. Cunningham, B., and Kirk, P. L., *J. Cellular Comp. Physiol.*, **20**, 119–34 (1942)
106. Tyler, A., and Berg, W., *Science*, **94**, 397–98 (1941)
107. Tobias, J., and Gerard, R. W., *Proc. Soc. Exptl. Biol. Med.*, **47**, 531–33 (1941)
108. Tobias, J., *Rev. Sci. Instruments*, **13**, 232–33 (1942)
109. Scholander, P. F., *Science*, **95**, 177–78 (1942)
110. Scholander, P. F., Edwards, G. A., and Irving, L., *J. Biol. Chem.*, **148**, 495–500 (1943)
111. Scholander, P. F., *Rev. Sci. Instruments*, **13**, 32–33 (1942)
112. Scholander, P. F., *Rev. Sci. Instruments*, **13**, 264–66 (1942)
113. Scholander, P. F., *J. Biol. Chem.*, **142**, 427–30 (1942)
114. Scholander, P. F., and Edwards, G. A., *Rev. Sci. Instruments*, **13**, 292–95 (1942)
115. Scholander, P. F., *Rev. Sci. Instruments*, **13**, 362–64 (1942)
116. Scholander, P. F., and Edwards, G. A., *Am. J. Physiol.*, **137**, 715–16 (1942)
117. Scholander, P. F., and Roughton, F. J. W., *J. Ind. Hyg. Toxicol.*, **24**, 218–21 (1942)
118. Roughton, F. J. W., and Scholander, P. F., *J. Biol. Chem.*, **148**, 541–50 (1943)
119. Scholander, P. F., and Roughton, F. J. W., *J. Biol. Chem.*, **148**, 551–63 (1943)
120. Edwards, G. A., Scholander, P. F., and Roughton, F. J. W., *J. Biol. Chem.*, **148**, 165–71 (1943)
121. Scholander, P. F., and Roughton, F. J. W., *J. Biol. Chem.*, **148**, 573–80 (1943)
122. Bodine, J. H., and Tahmisian, T. N., *Biol. Bull.*, **85**, 157–63 (1943)
123. Flexner, J. B., Flexner, L. B., and Straus, W. L., Jr., *J. Cellular Comp. Physiol.*, **18**, 355–68 (1941)
124. Romanoff, A. L., *J. Exptl. Zoöl.*, **93**, 1–26 (1943)
125. Friedenwald, J. S., Herrmann, H., and Buka, R., *Bull. Johns Hopkins Hosp.*, **70**, 1–13 (1942)
126. Herrmann, H., and Friedenwald, J. S., *Bull. Johns Hopkins Hosp.*, **70**, 14–18 (1942)
127. Rosenthal, O., Bowie, M. A., and Wagoner, G., *J. Cellular Comp. Physiol.*, **17**, 221–33 (1941)
128. Rosenthal, O., Bowie, M. A., and Wagoner, G., *J. Cellular Comp. Physiol.*, **19**, 15–28 (1942)
129. Berger, J., and Avery, G. S., Jr., *Am. J. Botany*, **30**, 290–97 (1943)
130. Berger, J., and Avery, G. S., Jr., *Am. J. Botany*, **30**, 297–302 (1943)
131. Berger, J., and Avery, G. S., Jr., *Science*, **98**, 454–55 (1943)
132. Lowry, O. H., *Biol. Symposia*, **10**, 233–45 (1943)
133. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, **143**, 257–69 (1942)

134. Lowry, O. H., Hastings, A. B., Hull, T. Z., and Brown, A. N., *J. Biol. Chem.*, **143**, 271–80 (1942)
135. Lowry, O. H., McCay, C. M., Hastings, A. B., and Brown, A. N., *J. Biol. Chem.*, **143**, 281–84 (1942)
136. Lowry, O. H., and Hastings, A. B., *Problems of Ageing*, 2nd Ed., pp. 728–55 (Williams & Wilkins Co., Baltimore, 1942)
137. Linderstrøm-Lang, K., *Nature*, **139**, 713 (1937)
138. Linderstrøm-Lang, K., and Lanz, H., Jr., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **21**, 315–38 (1938); *Mikrochim. Acta*, **3**, 210–30 (1938)
139. Holter, H., Lanz, H., Jr., and Linderstrøm-Lang, K., *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **23**, 1–9 (1938); *J. Cellular Comp. Physiol.*, **12**, 119–27 (1938)
140. Caspersson, T., *J. Roy. Microscop. Soc.*, **60**, 8–25 (1940)
141. Gersh, I., *Physiol. Revs.*, **21**, 242–66 (1941)
142. Mirsky, A. E., *Advances in Enzymology*, **3**, 1–34 (1943)
143. Gersh, I., and Caspersson, T., *Anat. Record*, **78**, 303–19 (1940)
144. Gersh, I., and Bodian, D., *Biol. Symposia*, **10**, 163–84 (1943)
145. Popper, H., *Proc. Soc. Exptl. Biol. Med.*, **43**, 133–36 (1940)
146. Popper, H., and Elsasser, M., *Can. J. Med. Tech.*, **3**, 45–50 (1941)
147. Popper, H., *Arch. Path.*, **31**, 766–802 (1941)
148. Popper, H., and Greenberg, R., *Arch. Path.*, **32**, 11–32 (1941)
149. Greenberg, R., and Popper, H., *J. Cellular Comp. Physiol.*, **18**, 269–72 (1941)
150. Greenberg, R., and Popper, H., *Am. J. Physiol.*, **134**, 114–18 (1941)
151. Popper, H., and Ragins, A. B., *Arch. Path.*, **32**, 258–71 (1941)
152. Cornbleet, T., and Popper, H., *Arch. Dermatol. Syphilol.*, **46**, 59–65 (1942)
153. Ragins, A. B., and Popper, H., *Arch. Path.*, **34**, 647–62 (1942)
154. Popper, H., Steigmann, F., and Dyniewicz, H. A., *Proc. Soc. Exptl. Biol. Med.*, **50**, 266–68 (1942)
155. Popper, H., and Chinn, H., *Proc. Soc. Exptl. Biol. Med.*, **49**, 202–4 (1942)
156. Popper, H., and Brenner, S., *J. Nutrition*, **23**, 431–43 (1942)
157. Meyer, K. A., Steigmann, F., Popper, H., and Walters, W. H., *Arch. Surg.*, **47**, 26–43 (1943)
158. Popper, H., Steigmann, F., Meyer, K. A., and Zevin, S. S., *Arch. Internal Med.*, **72**, 439–60 (1943)
159. Hirt, A., and Wimmer, K., *Klin. Wochschr.*, **18**, 765–67 (1939)
160. Hirt, A., and Wimmer, K., *Klin. Wochschr.*, **18**, 733–40 (1939)
161. Simpson, W. L., and Cramer, W., cited by Cowdry, E. V., *Biol. Symposia*, **10**, 156–58 (1943)
162. Marton, L., *Ann. Rev. Biochem.*, **12**, 582–614 (1943)
163. Schmitt, F. O., Hall, C. E., and Jakus, M. A., *Biol. Symposia*, **10**, 261–76 (1943)
164. Scott, G. H., and Packer, D. M., *Anat. Record*, **74**, 17–29, 31–45 (1939)
165. Scott, G. H., *Proc. Soc. Exptl. Biol. Med.*, **44**, 397–98 (1940)
166. Lansing, A. I., and Scott, G. H., *Anat. Record*, **84**, 91–96 (1942)
167. Cowdry, E. V., *Biol. Symposia*, **10**, 145–46 (1943)

168. Packer, D. M., and Scott, G. H., *J. Tech. Meth. Bull. Intern. Assoc. Med. Museums,* **22,** 85–96 (1942)
169. Scott, G. H., *Biol. Symposia,* **10,** 277–89 (1943)
170. Richards, A. G., Jr., Anderson, T. F., and Hance, R. T., *Proc. Soc. Exptl. Biol. Med.,* **51,** 148–52 (1942)
171. O'Brien, H. C., and McKinley, G. M., *Science,* **98,** 455–56 (1943)
172. Carruthers, C., *Ind. Eng. Chem., Anal. Ed.,* **15,** 70–71 (1943)
173. Carruthers, C., *Ind. Eng. Chem., Anal. Ed.,* **15,** 412–14 (1943)
174. Cowdry, E. V., *Biol. Symposia,* **10,** 149–50 (1943)
175. Laitinen, H. A., and Kolthoff, I. M., *Science,* **92,** 152–54 (1940)
176. Laitinen, H. A., and Kolthoff, I. M., *J. Phys. Chem.,* **45,** 1061–79, 1079–93 (1941)
177. Davies, P., and Brink, F., *Rev. Sci. Instruments,* **13,** 524–33 (1942)
178. Davies, P., and Brink, F., *Am. J. Physiol.,* (Proc.), **133,** 257–58 (1941)
179. Selzer, L., and Baumberger, J. P., *J. Cellular Comp. Physiol.,* **19,** 281–87 (1942)
180. DuBuy, H. G., and Olson, R. A., *Am. J. Botany,* **27,** 401–13 (1940)
181. Warren, C. O., *J. Cellular Comp. Physiol.,* **19,** 193–211 (1942)
182. Beecher, H. K., Follansbee, R., Murphy, A. J., and Craig, F. N., *J. Biol. Chem.,* **146,** 197–206 (1942)
183. Hoerr, N. L., Editor, *Biol. Symposia,* Vol. **10** (The Jaques Cattell Press, Lancaster, 1943)
184. Bensley, R. R., *Science,* **96,** 389–93 (1942)
185. Crossmon, G., *Science,* **85,** 250 (1937)
186. Stoneburg, C. A., *J. Biol. Chem.,* **129,** 189–96 (1939)
187. Marshak, A., *Science,* **92,** 460–61 (1940); *J. Gen. Physiol.,* **25,** 275–91 (1941)
188. Mirsky, A. E., and Pollister, W., *Biol. Symposia,* **10,** 247–60 (1943)
189. Laskowski, M., *Proc. Soc. Exptl. Biol. Med.,* **49,** 354–56 (1942)
190. Dounce, A. L., and Lan, T. H., *Science,* **97,** 584–85 (1943)
191. Dounce, A. L., and Seibel, D., *Proc. Soc. Exptl. Biol. Med.,* **54,** 22–24 (1943)
192. Lazarow, A., *J. Biol. Chem.,* **140,** lxxv (1941)
193. Dounce, A. L., *J. Biol. Chem.,* **147,** 685–98 (1943)
194. Lan, T. H., *J. Biol. Chem.,* **151,** 171–75 (1943)
195. Dounce, A. L., *J. Biol. Chem.,* **151,** 221–33 (1943)
196. Dounce, A. L., *J. Biol. Chem.,* **151,** 235–40 (1943)
197. Hoerr, N. L., *Biol. Symposia,* **10,** 185–231 (1943)
198. Zittle, C. A., and O'Dell, R. A., *J. Biol. Chem.,* **140,** 899–907 (1941)
199. Zittle, C. A., and Zitin, B., *J. Biol. Chem.,* **144,** 99–104 (1942)
200. Zittle, C. A., and Zitin, B., *J. Biol. Chem.,* **144,** 105–12, (1942)
201. Feulgen, R., Behrens, M., and Mahdihassan, S., *Z. physiol. Chem.,* **246,** 203–11 (1937)
202. Behrens, M., *Z. physiol. Chem.,* **258,** 27–32 (1939)
203. Williamson, M. B., and Gulick, A., *J. Cellular Comp. Physiol.,* **20,** 116–18 (1942)
204. Boell, E. J., Chambers, R., Glancy, E. A., and Stern, K. G., *Biol. Bull.,* **79,** 352 (1940)

205. Velick, S. F., *Biol. Bull.*, **81**, 303–4 (1941)
206. Hutchens, J. O., Kopac, M. J., and Krahl, M. E., *J. Cellular Comp. Physiol.*, **20**, 113–16 (1942)
207. Lazarow, A., and Barron, E. S. G., *Anat. Record*, **79**, Suppl. No. 2, 41 (1941)
208. Kabat, E. A., *Science*, **93**, 43–44 (1940)
209. Claude, A., *Biol. Symposia*, **10**, 124–26 (1943)
210. Stern, K. G., *Biol. Symposia*, **10**, 291–321 (1943)
211. Lazarow, A., *Anat. Record*, **84**, 31–50 (1942)
212. Mesch, A. C., *Biochem. J.*, **33**, 293–99 (1938)
213. Granick, S., *Am. J. Botany*, **25**, 558–61, 561–67 (1938)
214. Menke, W., *Z physiol. Chem.*, **257**, 43–48 (1938)
215. Mesch, A. C., *Biochem. J.*, **33**, 300–8 (1939)
216. Galston, A. W., *Am. J. Botany*, **30**, 331–34 (1943)
217. Hill, R., and Scarisbrick, R., *Nature*, **146**, 61–62 (1940); *Proc. Roy. Soc. (London), B*, **129**, 238–55 (1940)
218. French, C. S., and Anson, M. L. (In preparation)

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